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THE CELL

Volume IV

THE CELL

Biochemistry, Physiology, Morphology

Edited by

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New York, New York

VOLUME IV

Specialized Cells: Part 1

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PREFACE

The excellent reception accorded the first volume has been very encouraging. It is most rewarding for the contributors to realize that this treatise is filling a real need for a synthesis of up-to-date knowledge in the many active fields of research on the cell.

The chapters in these volumes deal with many different types of cells. Of course what one would desire is to have presented cells of the whole animate creation. "We ought not to hesitate nor to be abashed," said Aristotle, "but boldly to enter upon our researches concerning animals of every sort and kind, knowing that in not one of them is Nature or Beauty lacking." Selection, however, was necessary so that not all areas could be covered.

J. Brachet
A. E. Mirsky

July, 1960

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CHAPTER 1

Viruses

By RENÉ THOMAS

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I. INTRODUCTION

This chapter is by no means an attempt to review recent developments in virus research; the bibliography is far from extensive and, except for a few references added in proof, it ends at the beginning of 1958, when the paper was written. Our purpose is rather to outline some of the main research lines in the field of virology in connection with their bearing on general biology, and, in addition, to provide a minimal background of general information. Such important questions as, for instance, the relationships between cancers and viruses will not be treated here (see Oberling, Le Breton, and co-workers, in Volume V). Much information and stimulating discussions will be found in Luria's "General Virology" (1953) and recent review (1958). Most of the facts with which we shall deal are known from work on a very limited number of viral "species." A number of these facts have been demonstrated for the first time and most clearly for bacterial viruses. Partly for this reason, but also for personal ones, the present account will be biased towards bacterial viruses.

We first have to define our material, which is not an easy task in the case of viruses. The old definition based on the *size* of viruses is obviously insufficient by itself. It could hardly be expected that the limits of visibility with the light microscope or the filterability through bacterial filters could give more than an arbitrary, although often convenient, borderline between viruses and cells. In fact, some bacterial species and other microorganisms are smaller than the large viruses. Many bacteria of normal size can, under certain conditions, give rise to submicroscopic stages which can be reconverted into normal bacteria. On the other hand, some viruses (e.g., poxviruses) can be visualized with the light microscope after suitable coloration.

The most outstanding character common to all viruses is probably that they are *obligatory intracellular parasites of specific hosts.* This property is insufficient, however, to define a virus, since some small bacteria and other organisms are also intracellular parasites which have in many cases not yet been grown on acellular medium. On the other hand it is conceivable, although admittedly dubious, that further progress in the isolation of subcellular structures could finally enable us to grow viruses without the presence of intact cells.[1]

The relations between a virus and its host cell are more intimate than any other parasite-host relationship and should be situated at a different level of integration: increasing knowledge of the chemical composition of viruses shows that virus particles lack energy-yielding and synthetic enzyme systems, and in most or all cases lack one of the two classes of nucleic acids. It may be inferred that the need for a specific intracellular medium has here a deep significance and means more than just a group of complex nutritional requirements. When we look at viruses from this point of view the difficulty is no longer to distinguish them from cells, but rather from cell constituents. Viruses are, indeed, frequently compared to "normal" cell constituents, from which they are believed by many to have arisen.

---

[1] Bacterial protoplasts, on which phages grow readily, could hardly be considered as subcellular units, since they differ from ordinary bacterial cells only by the absence of cell wall constituents.

The distinction between viruses and cell constituents is not always an easy one to make and is probably sometimes meaningless. Especially interesting are the cases of the "kappa" factor in *Paramecium* (see Beale, 1954) and of the "génoïde" in *Drosophila* (see L'Héritier, 1957). The value of *pathogenicity* as a criterion is far from absolute, since many viruses multiply, at least in some hosts, without any harmful effect. As for the *transmissibility*, those normal cell constituents to which viruses are usually paralleled are also endowed with genetic continuity. In the case of these cell constituents, the transmission is achieved, in principle, from mother cell to daughter cells (although sexuality and related phenomena involve transfers that are sometimes very similar to contagion). On the contrary, viruses typically infect cells *from outside.* They are found not only in the cells, but also as extracellular, infectious but metabolically inactive particles. It is frequently the case, however, at least for bacterial viruses, that the viral genetic material associates with the host genome and adopts its rhythm of division. The disease is then carried as the inherited potentiality to produce virus particles along with immunity against infection from outside (provirus). If for some reason the ability to produce mature virus particles disappears, the viral genetic material may become hardly distinguishable from a host genetic marker conferring immunity (Jacob and Wollman, 1957).

Recent developments of outstanding importance indicate that, at least in the case of bacteriophages, virus particles are functionally differentiated into a genetic moiety which is introduced into the host cell and a coat which remains outside (Hershey and Chase, 1952). The coat is involved in the processes of fixation onto the cell and of injection of the genetic material. Once introduced in the cell, the latter—if not reduced to a provirus—turns to its own advantage the energy-yielding and synthetic systems of the host cell. It induces the synthesis of a number of nonhost substances, part of which will appear in the new viral particles. Recent evidence indicates that infection may be carried out, in the case of tobacco mosaic virus and of some animal viruses, using isolated genetic material (rather pure nucleic acid preparations).

For short, many viruses may be characterized as follows. They occur as submicroscopic infective particles, metabolically inert by themselves and lacking part of the fundamental constituents of a cell. These particles are able to invade specific host cells. This process involves, often and perhaps always, some kind of dissociation of the viral constituents. The viral genetic material then turns to its advantage the host cell synthetic abilities, and more virus is finally produced. Many viral forms, however, associate in a more or less symbiotic way with the host cell. The occurrence of mature particles and of infection from outside then becomes facultative and, in some cases, really exceptional. Such viral forms may be hard to distinguish from "normal" cell constituents.

To conclude this introduction, let us quote the definitions recently proposed by two outstanding virologists. ". . . whatever mechanisms future discovery may reveal, it seems now a fruitful unifying view to consider viruses as genetically specific cell constituents containing coded DNA or RNA which can, as one of their genetic functions, determine their own incorporation into specific vehicles for transmission to other cells" (Luria, 1958). "Viruses are complex organized infectious entities which can reproduce from their genetic material only" (Lwoff, 1959).

II. Properties of Virus Particles

A. Detection and Titration

The most obvious manifestation of viruses, in general, lies in the production of abnormalities in the host. This is the way that viruses usually are discovered. These symptoms will not be described in this paper, except for the important distinction between local and "systemic" (generalized) infections.

Local lesions are of special interest for the direct evaluation of the number of infectious units. This evaluation can be made for bacteriophages by plating an appropriate dilution of the virus preparation with an excess of sensitive bacteria on agar medium. After bacterial growth the phage particles that were present at the moment of plating can be scored as "plaques," discrete areas of growth inhibition. For animal viruses, the evaluation is often made by counting the number of lesions on an appropriate membrane, e.g., the chorioallantoic membrane of the chick embryo (Beveridge and Burnet, 1946). A more recent procedure is to count the number of plaques on a layer of tissue culture cells (Dulbecco, 1952). For plant viruses the determination is usually carried out by rubbing serial dilutions of the virus preparation onto susceptible plant leaves and scoring the number of lesions.

Generalized infections also are used, especially for animal virus titrations. The procedure followed is to determine what dilution of a virus preparation can give positive responses (end-point titration), or to count the number of positive and negative responses in groups of hosts, each group being treated with a different dilution of the virus preparation.

The method of plaque counts, when applied with dilutions of the same virus preparation, shows usually that the number of lesions varies as a linear function of the virus concentration. This observation indicates that a single material particle is enough to induce a lesion. This conclusion is confirmed by the fact that the distribution of the numbers of plaques produced by aliquots of a single suspension agrees with the distribution (Poisson) expected for independently distributed particles. Again the same conclusion comes out from the analysis of the frequency of positive responses as a function of dilution.

Each positive effect can thus be related to a single particle. Every virus particle however, for various reasons, will not succeed in establishing infection, so that the measured titer of infectious units will usually be proportional, rather than equal, to the real concentration of virus particles. The factor is frequently close to unity for bacteriophages, but it is often of the order of only 10^{-6} for plant viruses.

B. Morphology and Chemical Nature

When a viral particle is detected by any property other than the effects on the host, its identification needs special caution. The difficulties are similar to those encountered in proving that a given biological activity—for instance, enzymatic or transforming activity—is associated with a given molecule. Moreover it is known that different kinds of particles related to the same virus may be infectious. For instance, crystals of tobacco mosaic virus (TMV), TMV rods and various degradation forms of it, including ribonucleic acid (RNA) preparations, have been observed to be infectious. Particles homogeneous in size, shape, and properties have, however, been isolated in a number of cases and have been found to be always associated with a given viral disease, but lacking in the noninfected host. As their number is proportional, and in some cases almost equal, to the number of infective units, it is quite reasonable to call them virus particles.

Viruses are detected as individual particles either by optical methods (essentially electron microscopy) or by autoradiography of radioactive preparations (see page 27). A number of other physical techniques (ultrafiltration, sedimentation, diffusion, sedimentation equilibrium, light scattering, electrophoresis) have given valuable information about size and/or shape of virus particles. Some of them are essential tools for isolation and purification of viruses.

1. Bacterial Viruses (Bacteriophages, Phages)

The known bacteriophages appear as tadpole-shaped particles (Figs. 1 and 2), whose morphology is remarkably homogeneous for a given species. The head is more or less spherical or polyhedral, from about 100 mμ in diameter for the large ones down to about 20 mμ or less for the smallest.

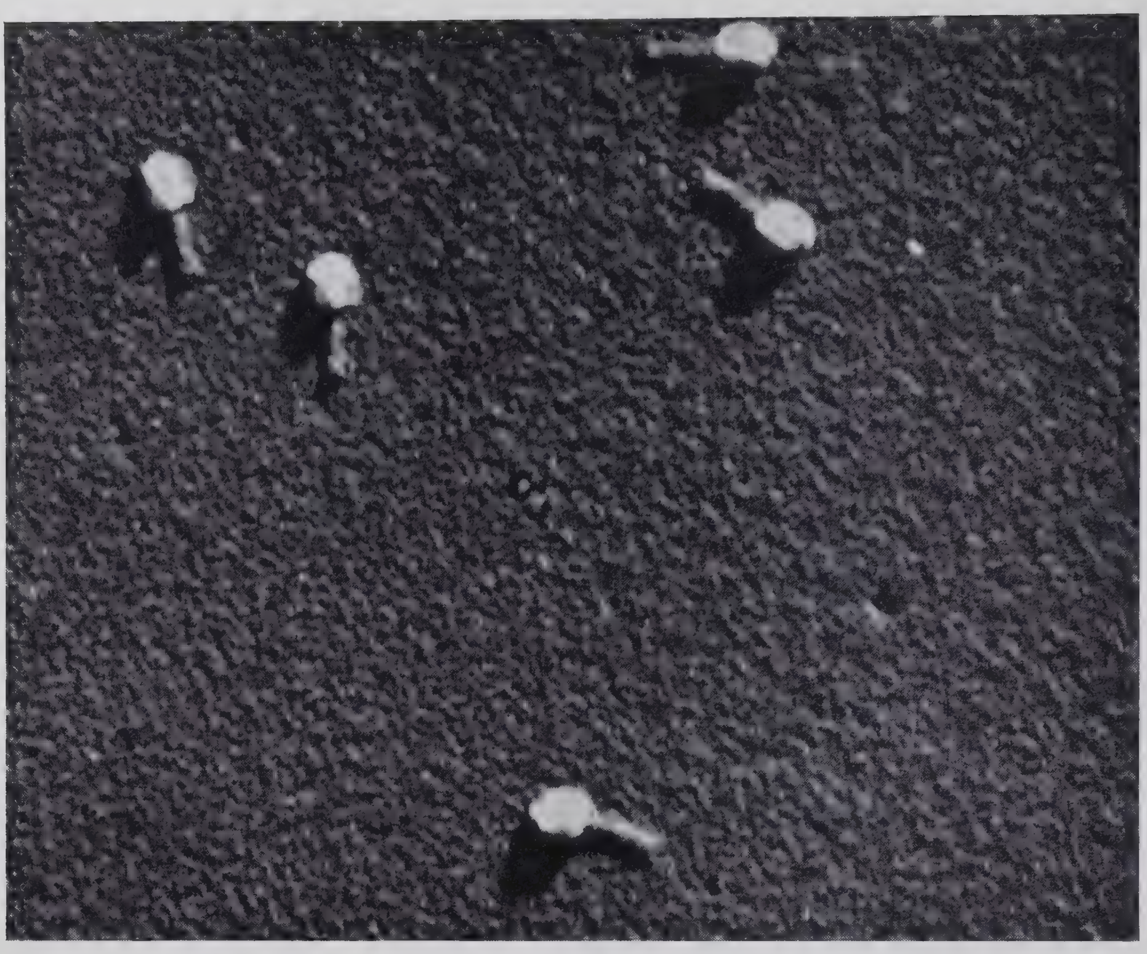

FIG. 1. Particles of T2 bacteriophages. Magnification: ×60,000. Electron micrograph by Dr. R. C. Williams.

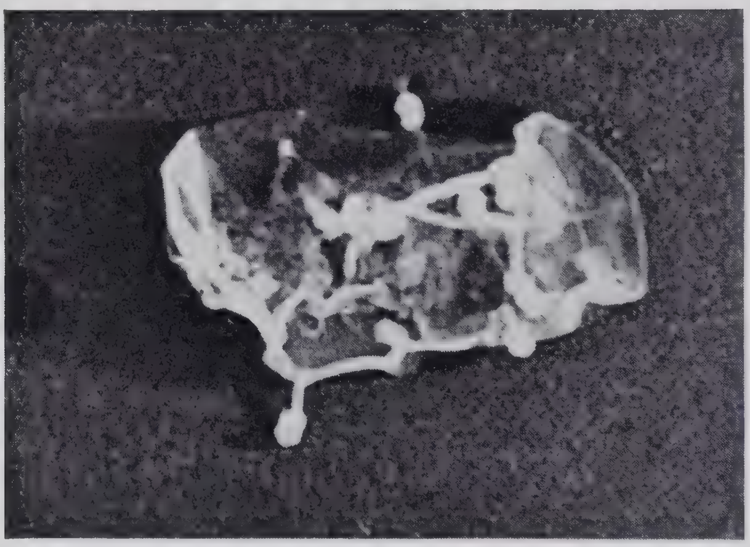

FIG. 2. T2 phage particles adsorbed on *Escherichia coli* membrane. Magnification: ×40,000. Electron micrograph by Dr. R. C. Williams.

The length and width of the tail vary depending on the type. The number of infective particles, as given by plaque counts, is in the best preparations close to the number of particles counted with the electron microscope (Luria *et al.*, 1951; Kellenberger and Arber, 1957).

Like other viruses, bacteriophages contain proteins and nucleic acid. Their nucleic acid is deoxyribonucleic acid (DNA) and is present in unusually high proportion, about 50%. Osmotic shock experiments (Anderson, 1949, 1953; Herriot, 1951) with phage T2 from *Escherichia coli* yield "ghosts" with normal morphology but empty heads, which are noninfectious (Fig. 3). During this treatment DNA is released from the phage particles. It is now sensitive to deoxyribonuclease (DNAase), unlike the DNA of intact phage particles. Most of the phage proteins sediment with the ghosts. The intact phage particle can thus be described in a simplified way as a DNA core enclosed in a protein membrane to which the tail is attached.

The most extensively studied phage, T2, contains 2×10^{-10} μg. of DNA (if it were all in a single molecule, it would correspond to a molecular weight of 1.2×10^8) whereas the small ϕX174 contains only about 70 times less DNA (corresponding to a molecular weight of only about 1.7×10^6) (Sinsheimer, 1957). The structure of most phage DNAs seems to be similar to other DNA preparations (X-ray diffraction pattern; four different nucleotides with the molar ratios: adenine $\approx$ thymine and cytosine $\approx$ guanine). It was found recently, however, that the small phage X174 contains a single-stranded DNA (Sinsheimer, 1959a, b). The so-called T-even phages of *E. coli* contain instead of cytosine, hydroxymethylcytosine (HMC) (Wyatt and Cohen, 1953); in part of the HMC nucleotides (hydroxymethyl deoxycytidylic acid) the nitrogen base is linked to one or two molecules of glucose (Sinsheimer, 1954, 1956; Volkin, 1954; Streisinger and Weigle, 1956).

The protein shell of phages can be dissociated by several methods, including freezing and thawing (Williams and Fraser, 1956). It is then possible to distinguish head membranes, intact tails, and tails degraded in various ways. The tail includes a central core surrounded by two protein sheaths, a proximal one and a distal one. The distal sheath is involved in the process of fixation on bacterial receptors, and, at least in T2, can be dissociated into several (four?) threads. The head membrane and the tail tip, at least, are antigenic, and the blockage of the latter by antiserum prevents phage adsorption. An enzyme, active on cell wall constituents, has been demonstrated in some cases (Kozinski and Opara, 1956; Kozloff *et al.*, 1957). In T2 this enzyme has the specificity of a lysozyme (Koch and Dreyer, 1958).

Besides external constituents and DNA, phage particles contain some

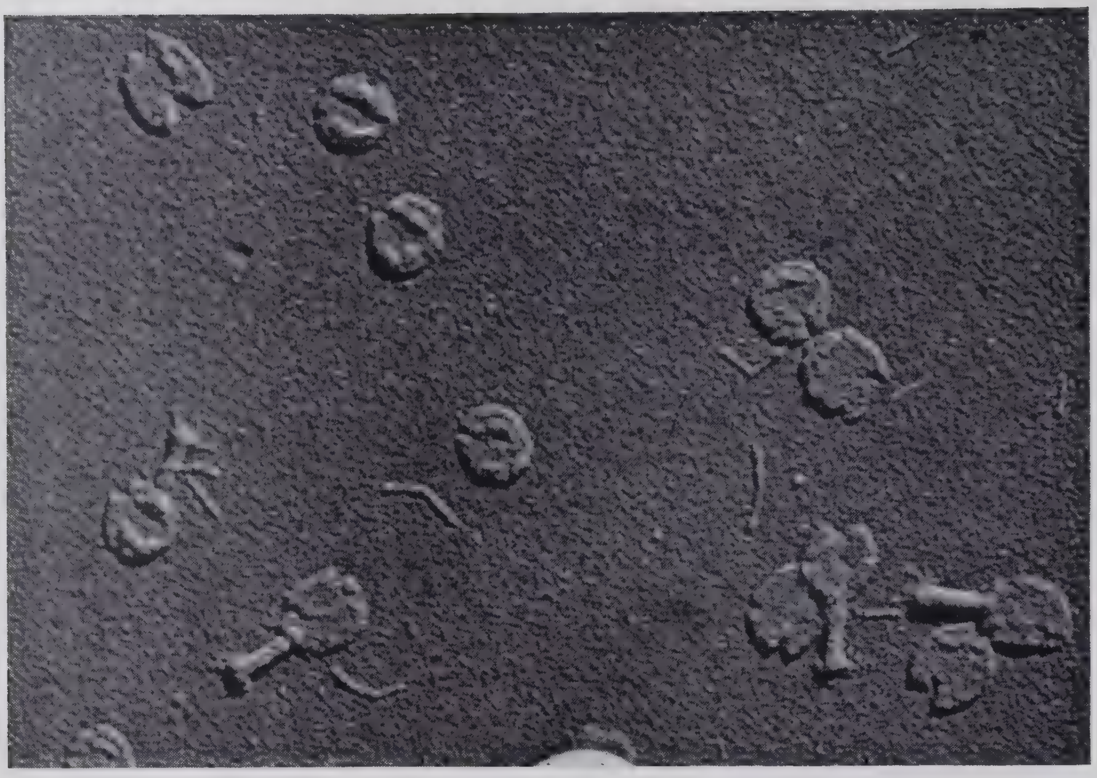

FIG. 3. T2 ghosts, head membranes, and tail fibers. Magnification: ×72,000. Electron micrograph by Dr. R. C. Williams.

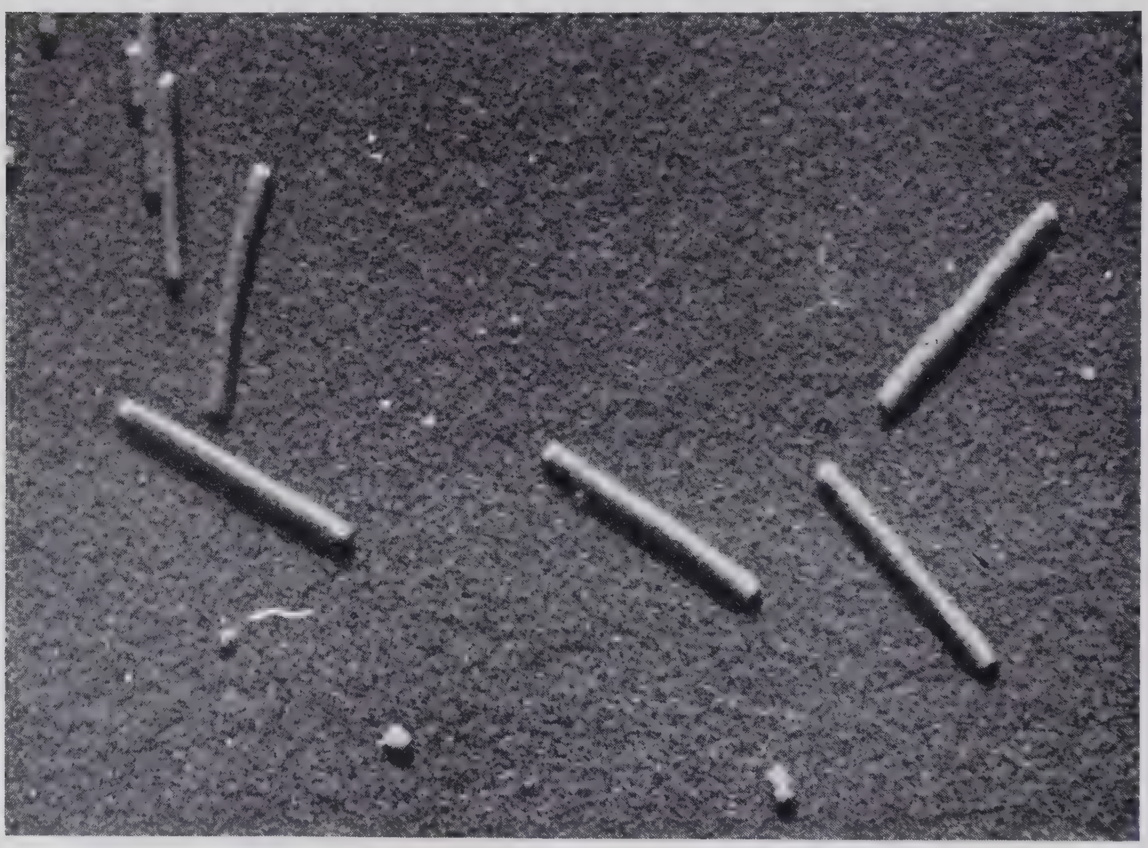

FIG. 4. Tobacco mosaic virus particles. Magnification: ×90,000. Electron micrograph by Dr. R. C. Williams.

minor internal constituents, which were investigated by Hershey (1955, 1957b) in order to determine whether DNA is associated with some other substances in phage "chromosome." Fractionation of labeled phage enabled him to characterize a "nonsedimentable" protein (in opposition to the other proteins, constituting the ghost), a peptide and two low molecular weight substances, which were later identified as polyamines (purescine and spermidine) by Ames *et al.* (1958).

Work of Volkin and Astrachan (1956b) excludes the presence of ribonucleic acid (RNA) in significant amounts.

Summary. Bacteriophages are, typically, tadpole-shaped particles containing DNA and proteins in about equal amounts. The DNA is enclosed in a shell (head membrane and tail) including several different proteins.

2. *Plant Viruses*

Plant viruses are either elongated rods [e.g., tobacco mosaic virus (TMV), 300 × 15 mμ] or small spheres, frequently about 20 mμ in diameter (e.g., turnip yellow mosaic virus). Figures 4 and 7 show both types of plant viruses (TMV and tomato bushy stunt virus) at the same magnification. In the case of rod-shaped viruses, various lengths are usually found in electron microscopic preparations. There is reason to think that they occur through fragmentation of particles of standard length or of their di- or trimers. Both types of plant viruses tend to crystallize. Some can be precipitated from purified suspensions into very regular crystals. The elongated type gives paracrystals, with parallel orientation of the rods, but without a regular three-dimentional network.

Plant viruses seem to be composed exclusively of ribonucleic acid and proteins. The relative amount of these two constituents is highly variable (6% RNA in TMV, 38% in turnip yellow mosaic virus), but the absolute amount of RNA per virus particle is rather uniform. The total RNA of a TMV particle corresponds to a molecular weight of about 2.5×10^6; it is now generally considered a single molecule rather than a group of subunits (Schramm and Gierer, 1957).

The protein moiety of TMV has been extensively studied and found to be composed of approximately 2300 probably identical polypeptide chains (Fraenkel-Conrat and Singer, 1954; Braunitzer, 1954, 1955; Schramm and Anderer, 1955). These polypeptide chains have a molecular weight of about 17,000. The polypeptide chains of different TMV strains have different terminal amino acid sequences (Nice and Fraenkel-Conrat, 1955).

As a first approximation, a TMV particle could be visualized as a central RNA string surrounded with a protein envelope made from a helical aggregation of the above-mentioned polypeptide molecules

Schramm *et al.*, 1955; Franklin, 1955a; Hart, 1955b, 1956). Very suggestive is the fact that viral particles partly degraded by alkali or by detergents appear on electron microscope pictures as fragments of rods with normal diameter, threaded on a central string (Fig. 5) which disappears following ribonuclease (RNAase) digestion. Some recent results, however, suggest that the above-described picture is oversimplified. Franklin *et al.* (1957) conclude from X-ray diffraction studies that, in intact virus particles, RNA is not really arranged as a central core, but rather as a coaxial coiled strand, with protein inside as well as outside (see also Ginoza, 1958). Schramm *et al.* (1955) already had observed that the central string of alkali-degraded TMV contains not only RNA, but also about 10% protein. Pirie (1956) showed that following heat denaturation TMV proteins are not entirely coagulated and that part of them remain associated with the RNA.

Interestingly enough, TMV proteins in some conditions spontaneously reaggregate into hollow rods of various lengths (Schramm and Zillig, 1955; Franklin, 1955b). When RNA from various sources—including Grünberg-Manago and Ochoa's (1955) synthetic polynucleotides (Hart and Smith, 1956)—is present, the protein reaggregates around the RNA into virus-like rods with RNA again protected against RNAase (Fig. 6). The important investigations concerning infectivity after reaggregation of TMV will be discussed below (page 21).

Less is known about the small spherical plant viruses (Fig. 7). It seems clear, however, that, here too, RNA is enclosed in a protein sheath (Markham, 1953).

Summary. Plant viruses are elongated rods, or more or less spherical particles. In both cases a ribonucleic core is protected by a protein sheath made of many, possibly identical, polypeptide molecules.

3. *Animal Viruses*

The size, shape, and composition of animal viruses are quite variable. Viruses of similar biological behavior are usually found to occur as morphologically similar particles ("elementary bodies") of similar composition. Elementary bodies are frequently more or less spherical or brick-shaped (Figs. 8–10)—about 450 mμ in diameter for psittacosis, 230 mμ for vaccinia (Fig. 10), 80 mμ for influenza (Fig. 8), 37 mμ for poliomyelitis. In some viruses filaments are found in addition to the spheres, but there are reasons to believe that they are noninfectious. Insect viruses which multiply in the cell nucleus are rods (about 280 × 40 mμ for polyhedrosis of silkworm). Cytoplasmic insect viruses are spherical or polyhedral. Some of them are as small as 12–15 mμ in diameter. A number of insect viruses, but not all (Smith, 1958), are found in intracellular inclusions, nuclear

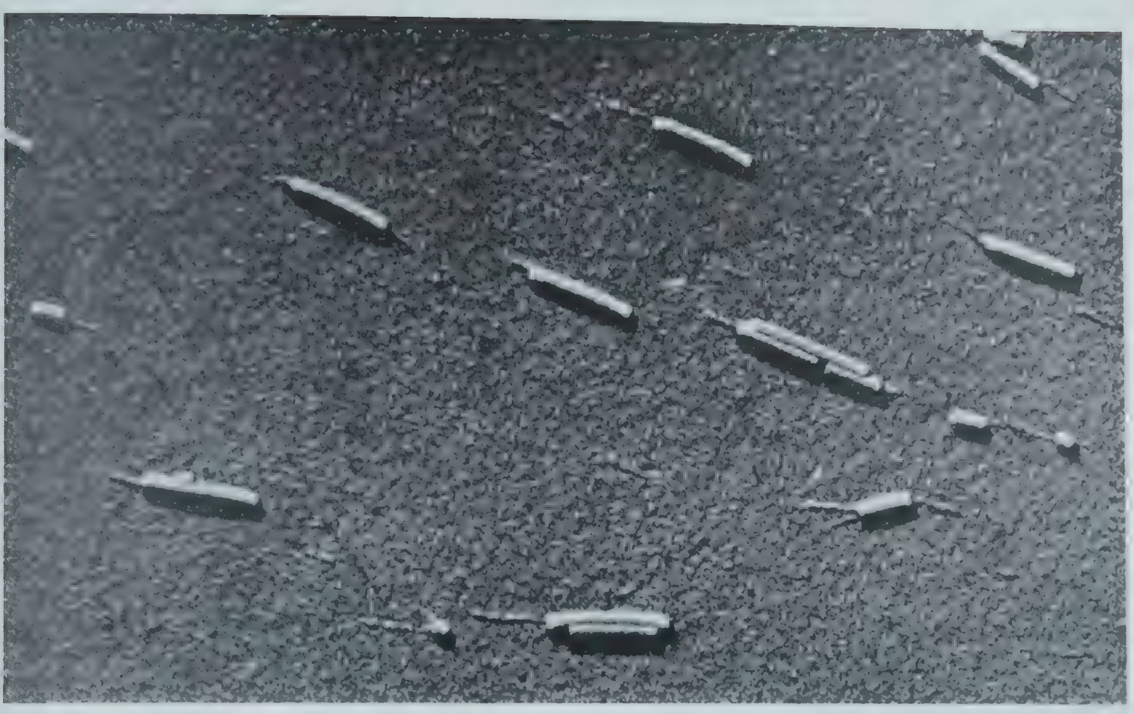

FIG. 5. Tobacco mosaic virus, degraded by detergent to show ribonucleic acid cores. Magnification: ×38,333. Electron micrograph by Dr. R. C. Williams.

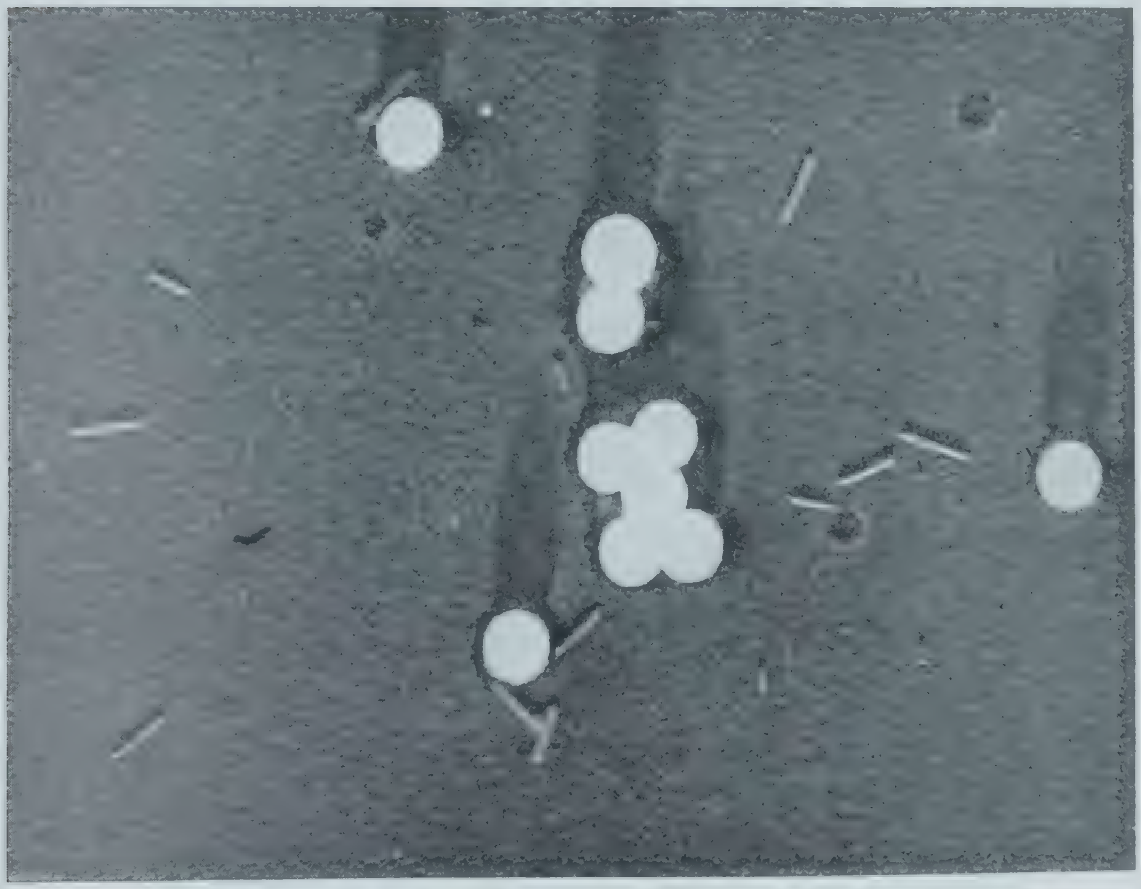

FIG. 6. Reconstituted tobacco mosaic virus containing both protein and nucleic acid. Magnification: ×27,275. Electron micrograph by Dr. R. C. Williams.

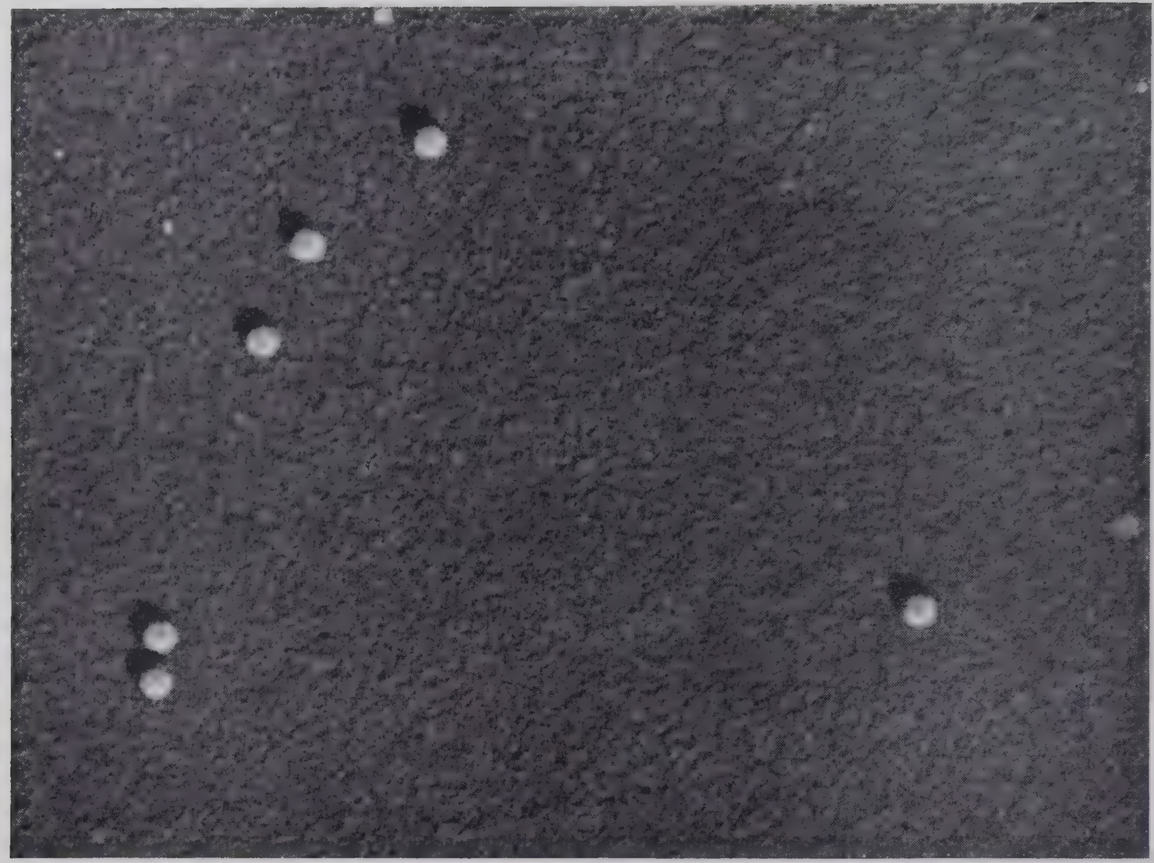

FIG. 7. Tomato bushy stunt virus, freeze-dried. Magnification: ×81,828. Electron micrograph by Dr. R. C. Williams.

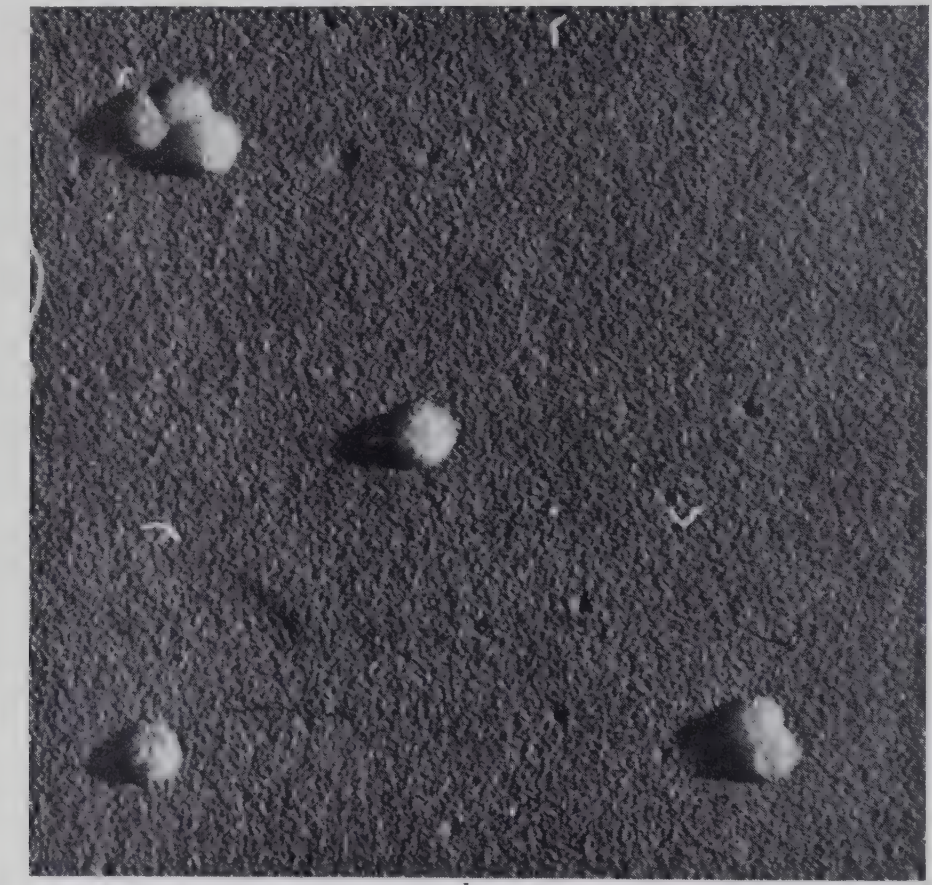

FIG. 8. Influenza virus. Magnification ×50,000. Electron micrograph by Dr. R. C. Williams.

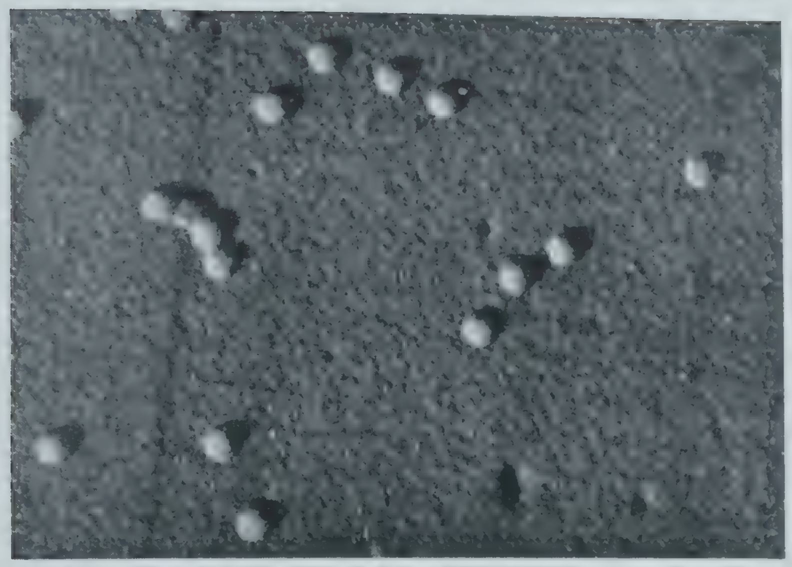

FIG. 9. Rabbit papilloma virus. Magnification: ×57,000. Electron micrograph by Dr. R. C. Williams.

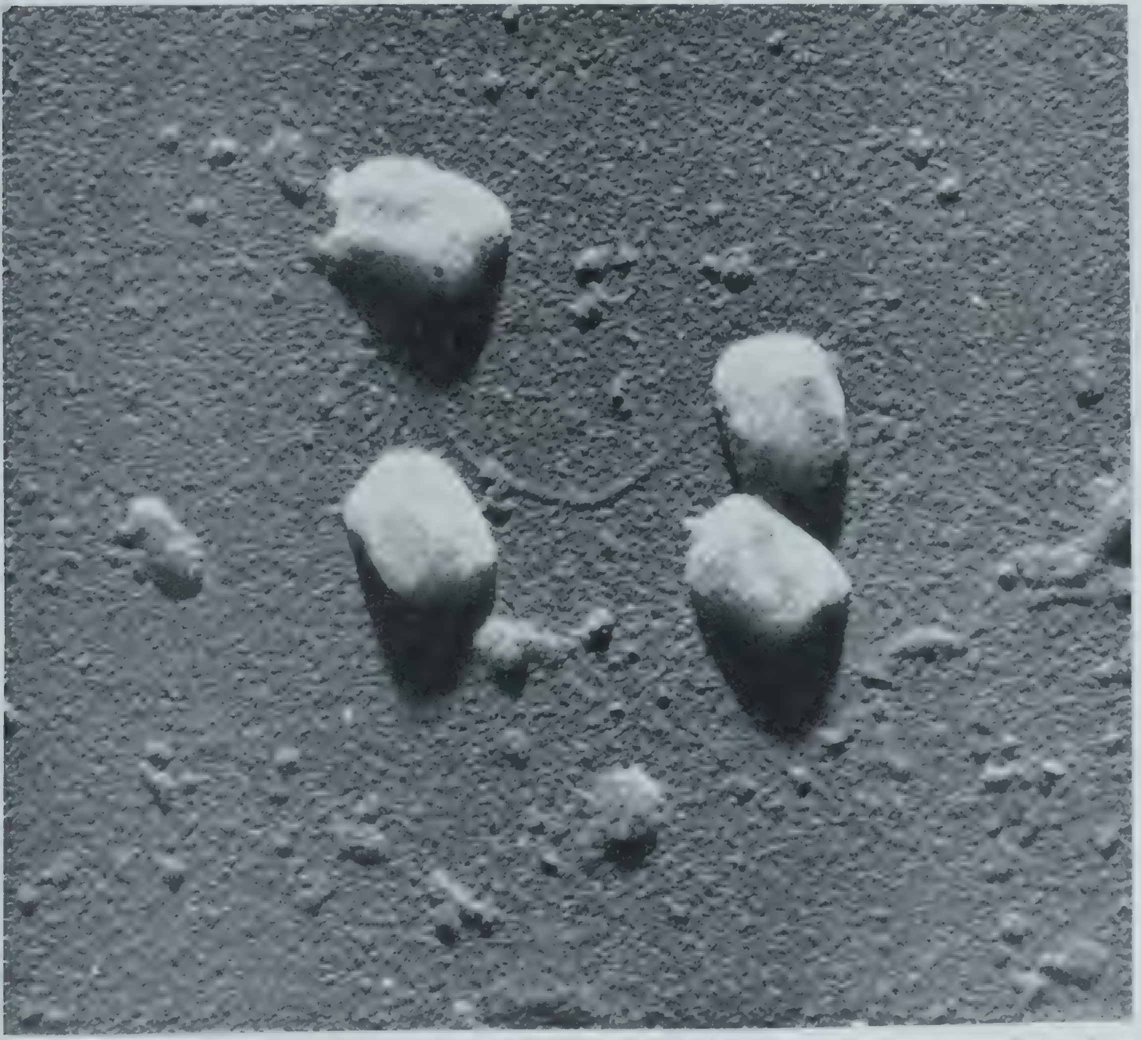

FIG. 10. Vaccinia virus. Magnification: ×45,000. Electron micrograph by Dr. R. C. Williams.

"polyhedra" or cytoplasmic "capsules." A table summarizing the size and morphology of the principal viruses can be found in Burnet's "Principles of Animal Virology" (1955). The poliomyelitis virus (Schaffer and Schwerdt, 1955), and an insect virus, the tipula iridescent virus (Williams and Smith, 1957) were recently crystallized.

The internal structure of animal viruses is less known than that of phages, as is their chemical constitution, which frequently is not restricted to proteins and a nucleic acid.

a. Nucleic acids. Pox-viruses (vaccinia group), papilloma and, probably, herpes, adeno-viruses and nuclear insect viruses, contain DNA. The so-called MNI (or myxo-) viruses (mumps, Newcastle disease, influenza, fowl plague), the poliomyelitis group, the equine encephalomyelitis group and the cytoplasmic insect viruses contain RNA. The psittacosis group may contain both types of nucleic acids.

b. Other constituents. Animal viruses exhibit a wide range of complexity. Poliomyelitis virus, for instance, contains only RNA and proteins, perhaps a single protein. Its complexity could be compared to that of plant viruses. Some other groups (e.g., pox- and MNI viruses), on the other hand, are very complex. MNI viruses contain not only nucleic acid and several types of antigenic proteins, but also a lipid-containing cement. These viruses have the property of hemagglutination (see below). Ether treatment releases noninfectious components: on the one hand, particles with hemagglutination activity and the major antigen including type, group, and strain specificities; on the other hand, ribonucleoproteic "soluble antigen" (Hoyle, 1952; Schäfer and Zillig, 1954). What follows is concerned with the MNI group viruses.

Hemagglutination was discovered by Hirst (1941, 1942), working with influenza virus. The virus can combine with red blood cells at low temperatures. If the mixture is incubated at 37° C., however, the virus is eluted, owing to the breakdown of the receptors on the cell surface by a viral enzyme. The red cells that have released an excess of virus become nonagglutinable, whereas the eluted virus is unchanged. Similar effects could be obtained, under proper conditions, with cells which allow the growth of the virus. It is now generally believed that the attachment of MNI viruses to host cells involves a combination between host cell receptor and viral enzyme. The requirements for attachment, however, are not so precise as for enzymatic activity, since the virus can still be absorbed if its enzyme has been inactivated. A number of animal fluids and tissue extracts have been found to inhibit the hemagglutination by viruses, at least when prior treatment has already suppressed the viral enzymatic activity. Apparently the inhibitors combine with the modified virus and prevent its combination with red blood cells (whereas intact virus hydrolyzes the inhibitors, yielding degradation products devoid of inhibitory power). The inhibitors have been identified as mucoproteins, and the cellular receptors for MNI group viruses are believed to have the same nature (Anderson *et al.*, 1948; Lanni and Théry-Lanni, 1955). Important progress was made possible by the discovery of a "model enzyme" in *Vibrio cholerae* extracts (Burnet, 1948). This enzyme (RDE: receptor-destroying enzyme) duplicates most of the virus effects on the red blood cells, including the disappearance of agglutinability by MNI group viruses. It hydrolyzes mucoproteins and destroys the activity of most of the agglutination inhibitors. The hydrolysis consists of a cleavage of the glycosidic bond joining the keto group of neuraminic acid to D-galactose or possibly other sugars. For this reason the name "neuraminidase" has been proposed for the specific enzyme of influenza virus and *Vibrio cholerae* (Gottschalk, 1957).

As reported above, small, noninfectious particles with hemagglutination activity are released by treatment of infectious particles with ether. Hemagglutinin actually consists of a complex mosaic of different antigens, some of which are probably more superficial, since they combine with the corresponding antibody *in vitro*, while others, more or less covered by the preceding ones, are detected only through inoculation *in vivo* under some special conditions. This complexity in composition and arrangement of the various antigens accounts for the numerous antigenic variants of influenza virus which occur in nature (Jensen and Francis, 1953).

The "soluble antigen" contains the viral RNA, associated with an antigenic protein (Hoyle, 1952; Ada and Perry, 1954). The characteristic antigenicity is revealed only when the particles are released from the virus, e.g., by ether treatment. This fact suggests that the soluble antigen particles lie inside the viral elementary bodies, like the nucleic acid of phages and plant viruses.

The lipid-containing, ether-sensitive fraction that holds together the constituents of MNI viruses includes, besides phospholipids, carbohydrates and proteins. This fraction has striking chemical similarities with some host-cell components, with which it cross-reacts serologically. It may result from the incorporation of elements of the cell surface into virus particles (for discussion, see Luria, 1959).

Summary. Animal virus particles vary widely in size, composition, and complexity. Most of them, perhaps all, contain *either* DNA *or* RNA. Some (e.g., poliomyelitis group) seem to contain only RNA and protein. Most are much more complex. The MNI group viruses contain (1) a ribonucleoprotein "soluble antigen," (2) a protein component with hemagglutination activity and various antigenicities, and (3) a complex lipid-containing cement.

III. MULTIPLICATION OF VIRUSES

A. Functional Differentiation of Constituents. Adsorption and Penetration of Viral Material into Host Cells

It was believed initially that virus particles are duplicated as such after penetration into the host cell. However, cytological evidence and the fact that, shortly after infection of allantoic membrane by influenza virus, most of the viral activity is lost (Henle and Henle, 1949) suggested that the virus does not exist as infectious particles during its intracellular multiplication. This "eclipse period" was most clearly demonstrated for bacteriophages (Doermann, 1952) and was later found to be the result of a functional differentiation of the viral constituents: as shown by Hershey and Chase (1952), the adsorption of bacteriophage T2 onto the host cell is followed by the penetration of only part of the viral constituents, essentially the genetic material.

1. Bacterial Viruses

a. Functional differentiation. "One-step growth" experiments (Ellis and Delbrück, 1939) permitted the isolation of a single cycle of phage growth. Although a number of variants of this technique have been published, it may be described in a simplified way as follows. Phage is mixed with a concentrated bacterial suspension in order to allow rapid adsorption. Unadsorbed phage is then removed by centrifugation or by using antiphage serum. The suspension is diluted to prevent resorption of the progeny phage particles. At intervals aliquots are plated with sensitive bacteria. At the beginning each infected bacterium, whatever its intracellular phage content, produces one plaque, so that the curve begins with a plateau showing the number of infected bacteria (latent period). As infected bacteria begin to lyse and liberate phage particles, a rapid increase in the number of plaques is found, reaching a second plateau when each infected cell is lysed.

If lysis of the cells is artificially induced (Doermann, 1952) during the second half of the latent period, many phage particles are liberated. If, however, the infected cells are lysed earlier, no phage particles are found, or, at least, much fewer than the number of infected cells. During multiplication the phage is thus in a noninfectious form known as the *vegetative phage.* The process by which mature infectious particles are formed from vegetative phage is called *maturation.*

The meaning of this eclipse period was not clear until it was realized that at the moment of infection the bacteriophage particle is dissociated into two parts, one of which remains normally adsorbed on the bacterial surface, whereas the other part is injected into the bacterial cell (Hershey and Chase, 1952). Using coliphage T2 labeled in its proteins with S^{35} and in its DNA with P^{32}, Hershey and Chase established that most of the phage protein remains at the outside of the bacterium and can be removed by violent shaking (Waring Blendor) without preventing growth of the phage. On the other hand, most of the phage DNA is retained by the cells even after shaking. Furthermore, whereas that part of parental phage S^{35} which penetrates into the cell is very poorly transferred to the progeny, as much as 70% of parental DNA P^{32} can be found in the progeny (Hershey, personal communication).

These results can be described as follows in morphological terms. The phage particle is adsorbed onto bacterial receptors by the tip of the tail (Anderson, 1953). Internal constituents, essentially DNA, are "injected" into the cell, whereas the "ghost" remains outside and can be stripped off without affecting phage multiplication.

The outer shell of a phage particle appears thus as a kind of syringe able to inject into the cell some phage material, which includes those constituents endowed with genetic continuity. This fraction is essentially DNA. It was very important, however, to exclude any minor constituent as possible genetic material. As reported above, subsequent work by Hershey (1955, 1957b) has led to the identification of internal minor constituents which, together with those constituents already known, account almost quantitatively for phage carbon. The study of the transfer of radioactive atoms of these minor fractions from parent to progeny phage shows that the transfer either is very low (nonsedimentable protein, peptide) or (polyamines) can be suppressed by competing with unlabeled polyamines.

As a whole DNA appears as the only substance whose transfer to progeny is effective and unavoidable for phage development. The work of Hershey and his collaborators provides, along with bacterial transformation data, the best present evidence for the genetic function of DNA.

b. Adsorption and penetration. Electron microscope pictures show that phage particles are adsorbed onto the bacterial cell wall by the tip of their tail (Anderson, 1953). More recent work (Kellenberger and Arber, 1955; Williams and Fraser, 1956) has shown that in the case of phage T2, the end of the tail is dissociated at the moment of adsorption into a series (four ?) of filaments which adhere to the bacterial cell wall. In T5, however, the fixation on bacterial receptors does not alter the morphology of the tail. The process of fixation is generally believed to include two successive steps, reversible and irreversible adsorption (Puck *et al.*, 1951) (see, however, the discussion in Hershey, 1957a). Fixation requires metallic ions, whose function is probably more specific than merely reducing the net negative charge of both bacterium and phage, since different phages require different ions. Other cofactors are sometimes necessary.

The irreversible attachment of a phage onto a bacterium is a highly specific process and is often compared to an antigen-antibody reaction. A given bacteriophage can often infect several related species (some phages, for instance, grow on *Escherichia, Salmonella,* and *Shigella* strains), but very closely related strains of the same bacterial species can be, one sensitive, the other resistant to a given phage. From a bacterial strain sensitive to a given phage, mutants can be isolated, differing from the wild type by a single mutational event, which no longer adsorb this phage (resistant strain[2]). It is possible then to isolate from the phage strain a mutant (host range mutant[3]) which is adsorbed by the bacterial mutant.

Bacterial receptors for a given phage must be spread all over the bacterial surface, since as many as two hundred T2 particles can be adsorbed by a single bacterium. An important step in the knowledge of these receptors was attained when Weidel and Kellenberger (1955) isolated from *Eschericha coli* spherical particles which were shown to combine in the ratio 1:1 with phage T5. From a mutant resistant to T5 similar particles were isolated which, however, do not adsorb on T5 particles.

Studies on the genetic control of host range and serological specificity[3] in hybrids between phages T2 and T4 gave results of the following type (Streisinger, 1956a, b). Crosses yield three phenotypically distinct types of phage particles. One is adsorbed on *E. coli* B/2 but not on B/4, and it is inactivated by serum anti-T4. The second is adsorbed on B/4, but not on B/2, and is inactivated by serum anti T2. The third is adsorbed on both B/2 and B/4 and is inactivated by both antisera. The results strongly suggest that the same structures are involved in the expression of both characters. At least two such structures (two protein molecules at the tail tip?) are present in each phage particle and are assembled at random with regard to each other and to genotype ("phenotypic mixing," see also page 25). A similar situation is found when the genetic control of the adsorption cofactor requirement is studied (Brenner, 1957).

The mechanism by which phage internal constituents are injected into bacteria begins to be known. Following attachment, the terminal portion of the T2 tail sheath spreads out into filaments; part of the central pin is exposed and may conceivably puncture the cell wall (Kellenberger and Arber, 1955; Williams and Fraser, 1956). The central pin is supposed to be injected into the bacterial cell, leaving the proximal half of the tail sheath with a hole through which the internal phage constituents might pass. Understanding from a more chemical point of view can be hoped, from development of the work of Rozloff *et al.* (1957, Kozloff and Lute, 1957a, b) with phages T2 and T4. The distal end of the virus tail can be removed *in vitro* by partial complexes of the zinc-group metals. This result is apparently due to the breakage of thiol ester bonds necessary to maintain normal structure of the virus tail. It seems that this reaction takes place when T2 and T4 phages are adsorbed on the *E. coli* cell wall (which contains zinc), resulting in the uncovering of an enzyme present in phage tail and able to digest the cell wall. It was shown, however, that even though there is an open passage through the altered viral tail, the release of viral DNA is dependent on temperature and the presence of compounds possessing a primary amino group.

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[2] If, for instance, from an *E. coli* strain B we isolate a mutant resistant to phage T2, it will be symbolized B/2. The "host range" mutant of T2 which is adsorbed on the strain B/2 is symbolized *h*, and by opposition the wild type is h^+. The latter will thus adsorb and grow on B but not on B/2, whereas *h* will adsorb and grow on both strains.

[3] As tested by the neutralization of phage infectivity by antisera. This serological specificity is apparently due to tail-tip antigens.

Summary. Phages are functionally differentiated into two major parts: genetic material—DNA—which is injected into the host cell, and a protein shell (including head membrane and tail) which remains outside. Attachment to a specific bacterial receptor is mediated by the tail tip. DNA injection involves complex processes, including the action of a phage enzyme on the bacterial cell wall.

2. *Plant Viruses*

Owing to the cuticle of plant epidermis, infection apparently can take place only if the virus is introduced inside the tissues or if superficial cells are damaged. Artificial infection can be produced with some viruses by rubbing the virus suspension on the leaves, with others by grafting or other means. Natural infection is frequently due to insects. In several cases it has been established beyond any doubt that plant viruses multiply, without harmful effects, inside the insect cells, and are transmitted from parent to progeny through the eggs. There is, however, no obligate cycle with alternate host, since it is possible to transfer these viruses indefinitely from plant to plant or from insect to insect. In both cases the virus particles remain unchanged and are able to infect again either host. These facts raise interesting questions concerning the specificity of the intracellular medium for viral growth.

In contrast with the field of bacteriophages, very few direct data are available about what happens at the moment of the infection of a cell by a plant virus particle. There are serious—although indirect reasons to think that the equivalent of the eclipse period does exist in plant virus infections. Although the resting TMV particles are insensitive toward RNAase, the infiltration of a solution of this enzyme in tobacco leaves within 6 hours after infection was found to prevent viral growth completely (Casterman and Jeener, 1955). This result is not due to formation of a complex of the viral particles with RNAase, since such a complex does not form at the ionic strength used. It is thus strongly suggested that some time after infection the RNA is dissociated from the protein coat and becomes accessible to RNAase action. Other data, including Luria-Latarjet type (Siegel and Wildman, 1956; Siegel *et al.*, 1957) and photoreactivation experiments (Harrison, 1956; Bawden, 1957) also point to some change in the state of viral material between infection and multiplication.

a. Functional differentiation. It is very remarkable to see how attempts to destroy the infectivity or modify the hereditary properties of plant viruses by mild modifications of the protein moiety have regularly failed. It is, for instance, possible to remove the terminal amino acids of each of the polypeptide molecules constituting the protein shell of TMV without producing any effect on infectivity or constitution of progeny protein.

On the other hand, it was found that several purine and pyrimidine analogs are powerful inhibitors of plant-virus growth. Their action was first explained as due to a simple competitive inhibition with the normal base. However, it was found later that the action of thiouracil, for instance, is only partly reversible by uracil: whereas the simultaneous addition of uracil completely suppresses the inhibitory action of thiouracil, the addition of uracil after thiouracil has been allowed to act alone for some time—say 2 hours—is almost ineffective, and the virus, in these conditions, multiplies only very slowly (Jeener, 1957).

This finding suggests that thiouracil inhibition is, at least partly, due to an incorporation of the abnormal base into viral RNA, giving thus an abnormal RNA, ineffective either for its own multiplication or for some stage of virus maturation. This hypothesis was tested in experiments with thiouracil labeled with S^{35} (Jeener and Rosseels, 1953), using a concentration of the inhibitor, which allows a small amount of virus to be synthesized. It was found that the abnormal base is indeed incorporated into the viral RNA, apparently as thiouridylic acid. As much as 5–18% of the uracil can be replaced by thiouracil. There is a close correlation between incorporation of thiouracil in the nucleic acid and inhibition of the viral growth. Incorporation of S^{35}-thiouracil into the nucleic acid is reduced by 90% in the presence of a concentration of uracil sufficient to suppress the inhibitory effect of thiouracil (Jeener, 1957). When TMV synthesized in the presence of thiouracil is assayed for infection, it is found that the amount of virus produced after a short time is drastically reduced as compared to that produced by infection with the same concentration of normal virus. Parallel work has been carried out by other investigators, notably by Commoner and Mercer (1952) and by Matthews (1953, 1956). Incorporation of abnormal bases into nucleic acids also has been observed in other material (e.g., bacteria, tumors). Bromouracil can replace as much as 70% of the thymine in phage T2. Concerning the mechanism of incorporation of abnormal bases, the results of *in vitro* work on synthesis of nucleosides with a liver phosphorylase (Friedkin, 1954; Friedkin and Roberts, 1954) should be remembered: many analogs of purines and pyrimidines are incorporated into nucleosides.

It is clear from the above-mentioned results that, in contrast to the failure of attempts to inactivate plant viruses by modifying their proteins, successful inhibition of the infectivity is obtained by modification of the RNA moiety. In addition, RNAase prevents viral growth if added early.

Direct evidence of the role of RNA in the establishment of infection was obtained recently in several laboratories. Gierer and Schramm (1956) and Fraenkel-Conrat (1956) succeeded in infecting tobacco leaves with RNA preparations obtained by phenol or detergent treatment of TMV. This important result was soon confirmed by others (Siegel *et al.*, 1957; Commoner, 1957; Bawden and Pirie, 1957). Although the method succeeds in extracting most of the RNA from the virus rods, the infectivity recovered from the preparations is very low as yet. Previous work had shown that TMV particles that have lost part of their proteins are still infectious (Schramm *et al.*, 1955; Hart, 1955a) while, on the other hand, Fraenkel-Conrat and Williams (1955), Lippincott and Commoner (1956), and Hart (1956) have obtained evidence of a substantial restoration of infectivity following reconstitution of viruslike particles from TMV-extracted RNA and protein. That in these reconstitution experiments RNA was actually the carrier of genetic information was suggested by the fact that when nucleic acid and protein from different strains of TMV are combined (Fraenkel-Conrat, 1956; Fraenkel-Conrat and Singer, 1957), the nature of the disease and the chemical composition of the progeny were generally similar to those of the strain supplying the nucleic acid.

The main evidence suggesting that the infectivity of the RNA preparations is actually due to RNA itself lies in the fact that, unlike TMV, the RNA preparations are inactivated by RNAase but not by anti-TMV immune serum. Bawden and Pirie (1957) excluded, on several grounds, that the infectivity could be attributed to intact TMV rods. They remark, however, that this activity is only a small fraction of that of the starting material, usually a fraction of a per cent. As the presence of a fraction of a per cent of protein was not excluded, the possibility remains, though unlikely, that the residual activity may be due to the presence of a small amount of material with the same activity, weight by weight, as intact rods. The interpretation of the results is further complicated by the fact that the effect of dilution and of varying the physiological state of the leaves is not the same on TMV and on RNA preparations. The low infectivity of RNA preparations may be due to the presence of RNAase in the leaves. The reconstitution of rods from TMV protein and RNA could simply protect the RNA against this action.

Summary. Rapidly increasing evidence that RNA is the genetic material of plant viruses is provided by inhibition experiments with RNA bases analogs and with RNAase, and by infection experiments with viral RNA preparations.

3. *Animal Viruses*

The occurrence of an "eclipse period" was first reported by Henle and Henle (1949) for influenza virus, and subsequently for a number of other animal viruses. It seems to be a general phenomenon, but it is only in some cases that it was rigorously demonstrated.

What components of animal viruses actually penetrate inside the host cell is far from clear. This question is currently being studied on influenza virus with methods of the sort used by Hershey for phages (Hoyle and Frisch-Niggemeyer, 1955; Hoyle and Finter, 1957; Wecker and Schäfer, 1957). Viral ribonucleic acid is presumably split from the bulk of the virus (or, at least, made sensitive to ribonuclease) early after infection with influenza, since viral growth can be prevented by ribonuclease (Leclerc, 1956).

Just as in the case of plant viruses, direct evidence of infection by viral RNA is rapidly accumulating. Infection could be carried out by Wecker and Schäfer (1957) for equine encephalomyelitis and by Colter *et al.* (1957) for Mengo encephalitis, using RNA preparations from infected cells. More recently Alexander *et al.* (1958) succeeded in infecting HeLa cells in a reproducible way with RNA preparations from partially purified poliovirus. The evidence that ribonucleic acid is actually the agent of the infection is the same as in the case of plant viruses.

B. Multiplication of Bacterial Viruses

1. *Biochemical Aspects*

Most of the biochemical data on phage growth come from work with phage T2. A very convenient characteristic of T2 (as well as of the other T-even phages) is that their DNA contains hydroxymethylcytosine (HMC) instead of cytosine (Wyatt and Cohen, 1953). Bacterial and viral DNA can thus be clearly distinguished in this case.

As discussed above, it can now be supposed that phage DNA injected at the moment of infection carries all the information lacking in the cell for the synthesis of new phage particles. This alone, of course, does not necessarily imply that the genetic information is, at any future point in phage multiplication, carried by DNA, or by DNA alone. For a discussion of this point, and evidence for a transfer of the information from DNA to another structure (ribonucleoprotein?) see the very attractive review of Stent (1958). On the other hand, see Jacob (1960) and Thomas (1959).

Dramatic changes in the cell metabolism take place at the moment of T2 infection. The synthesis of bacterial DNA is stopped. Phage DNA is rapidly synthesized from the following sources (see Hershey *et al.*, 1954):

(1) Parental phage is transferred to progeny with a high degree of efficiency [up to 70% of parental phage DNA can be found in progeny phages (Hershey, unpublished results)]. Different constituents of parental phage DNA are transferred with the same efficiency, and DNA precursors (including free bases and thymidine) do not interfere with the transfer. The parental DNA is thus not degraded to small building blocks.

(2) Bacterial DNA is used with high efficiency to synthesize phage DNA. In contrast with the transfer from parental phage to progeny phage DNA, the transfer from bacterial DNA is decreased to some extent if bases, nucleosides, or nucleotides are added as competitors.

(3) Other preformed bacterial sources (probably including RNA).

(4) Non-preassimilated sources.

Protein material related to the mature phage structures can be detected immunologically and with the electron microscope before mature particles are formed. It was shown in the case of an ultraviolet (UV)-induced lysogenic *Bacillus megatherium* that this material is synthesized later than DNA (Jeener, 1957). With T2, the synthesis of DNA and of phage protein could be almost completely dissociated by the addition of chloramphenicol about 10 minutes after phage infection (Melechen, 1955; Tomizawa and Sunakawa, 1956; Hershey and Melechen, 1957). DNA synthesis continues in the presence of chloramphenicol, whereas protein synthesis is reversibly blocked. It is possible to obtain phage particles in which almost all the DNA has been synthesized during the chloramphenicol period, whereas all the protein has been synthesized after removal of the antibiotic. These results show that phage DNA is not synthesized inside the phage protein coat. There is increasing evidence that this "chloramphenicol-DNA" is actually finished phage genetic material (Tomizawa, 1958; Thomas in Hershey *et al.*, 1958; Thomas, 1959).

It is interesting that this independence of DNA synthesis versus protein synthesis is not established immediately after infection: no DNA synthesis is observed if chloramphenicol or amino acid analogs are added just after infection (Burton, 1955; Melechen, 1955; Tomizawa and Sunakawa, 1956). It seems thus that proteins have to be synthesized in order to allow phage DNA synthesis to start. This hypothetical protein is usually referred to as the "early protein." In the case of T-even phages one thinks immediately of such proteins as the enzyme which converts deoxycytidylic acid into hydroxymethyldeoxycytidylic acid (HMC nucleotide). This, however, is not the whole story, since phage that contain cytosine in their DNA also need some minutes between infection and the addition of chloramphenicol in order to be able to synthesize DNA. Furthermore Gros and Gros-Doulcet (1958) observed the same kind of phenomenon for the resumption of DNA synthesis in *Escherichia coli* mutants requiring an amino acid: after a starvation for the required metabolite, followed by its addition, DNA can be synthesized in the presence of chloramphenicol, but only to an extent that depends on the time that elapsed between the amino acid supply and the addition of the antibiotic.

It was also shown by Neidhardt and Gros (1957) in *E. coli* that chloramphenicol inhibition of protein synthesis is linked to the synthesis of an abnormal RNA, so that the prevention by chloramphenicol of both "early protein" and phage-precursor proteins might well be related finally to an effect on RNA. That RNA actually intervenes in phage growth was first suggested by the discovery (Hershey, 1953) of an RNA fraction with high metabolic activity, after T2 infection. This RNA has base ratios different from those of total bacterial RNA and closer to those of phage T2 DNA (Volkin and Astrachan, 1956a). Also Jeener (1958) showed that phage multiplication following induction of a lysogenic strain of *Bacillus megatherium* can be prevented by RNAase and by 2-thiouracil. Such results are not entirely unexpected, since it has been known for several years (Brachet, 1941, 1955; Caspersson, 1941; Gale, 1956; Spiegelman, 1956) that RNA plays a key role in protein synthesis. That the participation of RNA in protein synthesis also holds true for organisms like phages, whose mature form does not contain RNA, would nevertheless be of primary interest.

Many bacterial enzymes continue to function, although most of them no longer appear to be synthesized after T2 infection: for instance, respiration continues at the same rate as at the moment of infection (Cohen, and Anderson, 1946). However protein synthesis continues at the same rate as just before infection, and only a small percentage of it is phage-precursor protein (Hershey *et al.*, 1954). It may be supposed that at least part of those proteins that are synthesized after phage infection, but are not incorporated into phage particles, can be accounted for by: (1) Proteins which are present neither in uninfected bacteria nor in mature phage, but which function in viral growth or release. These substances are presumably synthesized under control of viral genes and, if so, should be considered as constituents of the intracellular virus, in spite of the fact that they are never included in mature phage particles. To this class belong, for instance: the enzyme that makes HMC nucleotide from deoxycytidylic acid (Flaks and Cohen, 1957); the system that allows thymineless mutants of *E. coli* to synthesize thymine for phage DNA when infected with T2 (Barner and Cohen, 1954); and perhaps the *endolysins* (Jacob and Fuerst, 1958). (2) Substances whose function in phage growth or release is not obvious but which appear as a result of phage infection (see Section IV, D: Conversion).

As reported above, phage-related structures can be seen by electron microscopy when infected cells are prematurely lysed (Kellenberger and Séchaud, 1957; Kellenberger and Kellenberger, 1957). These structures are empty heads, tail cores, and fibers that are probably phage DNA molecules. As the authors point out, the membranes might have been either empty inside the cells or they might have arisen as an artifact of lysis, resulting from a release of DNA during the time of manipulation, because the immature coat was still fragile.

Although the phage proteins are obviously synthesized under genetic control of the phage DNA, the protein molecules that form the coat of a given phage particle are apparently synthesized independently of each other and of the DNA molecule they protect. This is, at least, the most reasonable explanation of results concerning *phenotypic mixing* (see principally Streisinger, 1956a, b): after mixed infection with two closely related phages differing by a coat-linked character, the progeny contains particles with either (or both) phenotype(s) associated, apparently at random, with either genotype (see also page 18).

An enzyme produced under phage control, "endolysin" (Jacob and Fuerst, 1958), appears in the middle of the latent period and increases until lysis. It acts on a component of the bacterial cell wall and transforms the cells into protoplastlike spheres, which almost immediately burst unless the medium is hypertonic. The whole phage yield of each cell is thus released at once.

2. *Genetic Aspects*

Spontaneous, as well as induced, mutations occur in bacteriophages (Luria, 1945; Delbrück and Bailey, 1946; Hershey, 1946). They take place during the intracellular phage multiplication, so that those cells which yield mutants usually contain, besides the mutants, many more nonmutated particles. An essential step in the understanding of phage growth was provided by a study by Luria (1951) of the frequency distribution of spontaneous phage mutants liberated by the lysis of individual bacteria. The mutants are not distributed at random, but as clones of variable size. This indicates that the mechanism of multiplication of phage genetic material must be geometric, the replicas formed from the infecting genomes being themselves able to reproduce.

When a bacterium is infected with two or more phages which are closely related but differ by at least two genetic markers, part of the progeny consists of phage particles which have inherited markers from two or more parents (Delbrück and Bailey, 1946; Hershey, 1946; Hershey and Rotman, 1949). The study of the frequency of recombination between markers enabled phage geneticists to draw linkage maps for phages as had been done previously for higher organisms. All data point to a linear arrangement of genetic markers (Hershey and Rotman, 1949; Benzer, 1955). The known markers of the temperate phage λ (Wollman and Jacob, 1954) and, more recently, of other phages, have been found to be on a single linkage group.

That recombination occurs before maturation of the phage particles appears from the fact that a substantial amount of recombinants is already found in the very first mature phage particles isolated after premature lysis (Doermann, 1953). Analysis of the recombination frequencies led to the quantitative theory of Visconti and Delbrück (1953), based on the idea that recombination occurs from mating at random of particles in a vegetative pool, from which particles are withdrawn at random for maturation.

It is known from the work of Hershey and Chase (1951) that T2 particles arising from a mixed infection contain an appreciable number of particles which segregate both types of progeny, and are thus heterozygotes. Since, for each marker studied, about 1 to 2% of the particles are found to be heterozygous, it might be inferred that most of the particles in a phage population have a short diploid region. Levinthal was able to show that the heterozygote genomes are made up of two partial genomes, one of each parent type, with a short overlapping region. They could arise from the joining of two "partial replicas" (Hershey, 1952), initiated at opposite ends of two homologous paired genomes. Levinthal also showed that the formation of heterozygotes may account for the production of recombinants.

Whatever the precise way in which phage recombinants arise, it is now quite widely believed to involve some "partial replica" (Hershey,

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[4] One of the most convincing arguments is the fact that after infection with one particle of unirradiated phage and several particles of a different, irradiated, parent (conditions which force early and multiple recombination involving the minority parent), the latter nevertheless survives as a clone (Jacob and Wollman, 1955).

1952) mechanism or "copy choice" (Lederberg, 1955).[4] These models have in common the belief that recombination between two (or eventually more) genomes is effected by the synthesis of a new genome under "cooperative control" of the parents, which remain intact. Recombination would thus be associated with duplication of the genetic material and would not involve "physical breakage" of the parent strands.

As phage DNA is, without doubt, the best candidate for "vegetative phage," it is essential to know something about the behaviour of individual DNA molecules during phage multiplication. Most spectacular information in this field is provided by the work of Levinthal (1956), who applied track autoradiography (Ficq *et al.*, 1954; Levi, 1954; see also Chapter 3 by Ficq in Volume 1) to the study of P^{32}-labeled phage DNA. When individual particles, each containing several atoms of P^{32}, are allowed to decay in a suitable photographic emulsion, tracks, due each to the disintegration of one P^{32} atom, are distributed as "stars," each centered on a labeled particle. Scoring of the tracks emerging from each star, together with the knowledge of the specific radioactivity of the material and of the time of decay, allows an estimation of the mass of DNA in each particle. When this procedure is applied to intact phages, it gives a determination of the DNA content of phage which is in reasonable agreement with other determinations. When applied after osmotic shock it gives, per phage, one large particle of DNA, about 50×16^6 in molecular weight, or 40 % of the phage DNA. The remainder of the DNA seems to be made up of rather small particles, as they do not appear as stars. If the labeled phage particles are used for infection and the progeny is studied in the same way, it is found that about one large labeled particle, about 25×10^6 in molecular weight, is recovered per parental phage. The same large particle is found after a second generation. If the large DNA particle found after osmotic shock can be identified with phage genome, these experiments eliminate the idea that extensive dispersion of the atoms of genetic material takes place during duplication or recombination. The data can be accounted for by the type of duplication models first suggested by Watson and Crick, 1953).

C. *Multiplication of Plant Viruses*

We discussed above evidence for the ribonucleic acid nature of plant virus genetic material and for the occurrence of what seems to be an "eclipse period" following infection. It is not known whether only RNA, or more of the viral material, is introduced into the host cell, although it is suggested that RNA is split from the protein, or, at least, made accessible to RNAase. Increase in UV resistance of the infective centers takes more time after infection with TMV than after infection with RNA preparations from TMV. This difference could be due to the time needed for TMV splitting (Siegel *et al.*, 1957); the authors of this work suggest that infection with RNA occurs in natural conditions, especially for transmission of infection from cell to cell.

Some data are now available concerning RNA synthesis after infection. A UV-microspectrographic study by Zech and Vogt-Köhne (1955) has shown that nucleic acid (presumably RNA) increases, first in the nucleus, then in the cytoplasm, during the early postinfection period. Accumulation of RNA in the infected tobacco leaf before the increase in infectious virus was also shown by Basler and Commoner (1956) and by Schramm (1958).

More information has been published concerning the appearance of virus-related antigenic proteins. Bawden and Pirie (1945) have shown that TMV-infected leaves contain various constituents with the same immunological specificity but with different rates of sedimentation. The smaller particles are noninfective. Markham and co-workers (review: 1953) have demonstrated that turnip yellow mosaic virus purified by successive crystallizations contains two kinds of particles, with different molecular weights and densities, which can be separated by centrifugation. Both types of particles have the same dimensions and shape, and give crystals having the same appearance and structure. The difference between the two types of particles lies in the fact that one contains 38% RNA and is infectious, whereas the other is empty and noninfectious. The latter might be compared to the "doughnuts" or to the ghosts of phage T2. TMV-infected plants contain protein constituents with very low sedimentation rates ("soluble antigens" of Jeener). These proteins are serologically related to the virus (Takahashi and Ishii, 1952; Jeener and Lemoine, 1952; Jeener *et al.*, 1954). They contain little or no RNA and are noninfectious. They are irreversibly aggregated by the techniques used for purification (precipitation by ammonium sulfate or by controlled pH lowering), as shown by the fact that when they are redissolved after purification, they can easily be sedimented by ultracentrifugation. Further aggregation leads to the formation of protein rods similar to viral particles (Rich *et al.*, 1955), and lowering the pH to 3.4 causes complete precipitation as paracrystalline needles identical in form with virus paracrystals (Jeener *et al.*, 1954).

The amount of "soluble antigen" in infected leaves is always very low. However, tracer experiments show that CO_2 is incorporated much faster into the soluble antigen than into the virus and into the normal leaf proteins. The experimental increase of viral specific activity can be accounted for quantitatively by the hypothesis that the soluble antigen is the only protein precursor of the virus (Jeener, 1956). The formation of a TMV particle could be imagined as the aggregation around the ribonucleic (or ribonucleoprotein) core, of many protein building blocks taken from the soluble-antigen pool. If this maturation process may be compared to the aggregation of TMV protein around RNA *in vitro*, it may be expected to be rather unspecific and to lead to some kind of phenotypic mixing.

D. Multiplication of Animal Viruses

No general scheme can be outlined as yet for the intracellular growth of animal viruses.[5] The above-mentioned occurrence of an "eclipse period" was reported for RNA-containing viruses as well as for such DNA-containing viruses as vaccinia and herpes. In the case of psittacosis virus, the eclipse period can be extended for a period as long as 13 days, if the cells have been previously submitted to starvation and are maintained in a minimal, insufficient medium (e.g., Hanks' solution); when this medium is supplied with the necessary nutritive material, the infectious cycle is resumed, starting from the very stage where it was stopped. After a latent period of a few hours, an increasing number of complete particles appear in the cells and are then normally released into the medium (Morgan, 1956; Schiptt and Morgan, 1957). Whether or not the whole virus particle penetrates inside the host cell is not known for sure. That some kind of splitting of the viral constituents occurs early after infection is strongly suggested by the fact that viral multiplication, in the case of influenza, is RNAase-sensitive (Leclerc, 1956), and also by the work of Hoyle and his collaborators (Hoyle and Frisch-Niggermeyer, 1955; Hoyle and Finter, 1957).

In the case of the MNI group viruses, the early period is characterized by the successive appearance of the following, noninfectious, viral constituents:

(1) RNA-containing "soluble antigen."[6] This precursor, the first to be detected, is apparently identical with the "soluble antigen" which is found after ether treatment of mature virus particles.

(2) Particles with hemagglutinating activity (see Section II, B, 3). These particles appear later than does the soluble antigen and accumulate in the cells before the infectivity develops (Henle and Henle, 1949). They are (in contrast with the hemagglutinating particles which are separable after ether treatment of mature virus) frequently found as large particles, similar to complete virus but lacking RNA and infectivity, or as long filaments. It is not yet quite clear which of these structures

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[5] For a recent, short review, see for instance Sanders (1957)

[6] The "soluble antigen" of animal viruses is not homologous with Jeener's "soluble antigen" from infected plant cells. The first one is the RNA-containing moiety, and thus, presumably, the genetic moiety of some animal viruses; the other is the precursor of the protein (somatic) moiety of plant viruses.

are true precursors and side products of the virus. Incomplete infectious cycles, leading to the formation of "empty" influenza virus particles, in which the hemagglutinin does not envelope any ribonucleoprotein, have been reported in two distinct cases: infection of some nonsusceptible host (Schlesinger, 1950) or infection of susceptible hosts with a high concentration of virus (von Magnus, 1952). In the latter case, the abortive infection seems to be due to a shortage of some material present in the surrounding medium and used by the cell for cementing the hemagglutinin to the ribonucleoprotein.

Only scattered information is available as concerns the *association of vegetative virus with cell organelles*. Herpes, which is probably a DNA-containing virus, apparently multiplies within the nucleus of the infected cells: this was made clear by electron micrographs (Morgan *et al.*, 1954) and by work using fluorescent antibody (Lebrun, 1956, 1957). The same conclusion was reached for adenoviruses, which presumably also contain DNA. Insect viruses apparently multiply in the nucleus or in the cytoplasm according to whether they belong to the DNA or to the RNA group. Mumps and influenza antigens are first found in the cytoplasm of the infected cells, the latter close to the nuclear membrane (Watson, 1952; Liu, 1955). In the case of poliovirus, the presence of viral antigen is detectable in the cytoplasm 1 hour after infection, and it seems to occur in the nucleus only as a consequence of nuclear alteration (Lebrun, 1957).

Maturation in influenza virus takes place near the surface of the host cell. The soluble antigen and hemagglutinin particles are embedded in a lipid-containing cement, which might well be derived from the cell surface. In the case of herpes virus, maturation appears to take place as viral constituents migrate from the nucleus to the cytoplasm.

The *release* of animal virsuses from the host cell looks rather different from that of bacteriophages (see page 25). In the case of those animal viruses which have been studied in detail in this connection, the infected cells are able to secrete virus particles for a long period, apparently without serious damage to the cell surface. Individual poliomyelitis-infected cells, for instance, release virus particles for hours (Lwoff *et al.*, 1955). In chorioallantoic cells infected with influenza virus, new virus begins to appear after a latent period of 2–3 hours and is subsequently released at a nearly constant rate for up to 30 hours or even longer. Destruction of cell receptors by RDE (see Section II, B, 3) inhibits the adsorption of virus, but if added after penetration it hastens the release of the mature particles; on the other hand, blockage of host receptors with antiserum inhibits the release as well as the penetration of influenza virus (Quersin-Thiry, 1955). For some groups of viruses, the release into the medium is very poor: only 2–6% of the total amount of virus is found outside the cells, even on the sixth day after infection, in the case of adenoviruses (Ginsberg, 1958). High doses of these viruses cause an early, special, cytopathic effect, induced by a toxinlike material which is separable from the virus particles by ultracentrifugation, neutralized by antivirus serum, and destroyed by trypsin (Pereira, 1958; Everett and Ginsberg, 1958).

The relationship between virus production and cytopathic alterations is not clear. It is, for instance, possible to reverse in some cases the cytopathic effects without preventing virus formation, or vice versa. Fluorophenylalanine, which stops poliovirus growth at an early stage, does not interfere with the development of the cytopathic effects in HeLa cell cultures (Ackermann *et al.*, 1954). Other viruses, e.g., L'Héritier's *génoïde* (see, for instance, L'Héritier, 1957), do not induce any morphological change.

Genetic recombination between strains of influenza virus differing by more than one pair of characters was demonstrated first by Burnet and Edney (1951). Cross reactivation has also been described (Baron and Jensen, 1955; Hirst and Gotlieb, 1955).

The recent achievement of techniques (Dulbecco, 1952; Cieciura *et al.*, 1957; Lwoff, 1959) which enable us to study infection of animal cells *in vitro* is obviously of primary interest for the understanding of viral diseases. It may be argued that the situation in a diseased animal is so much more complicated than *in vitro*, that the study of infected cells *in vitro* is of little practical value. The prominent role of such simple, nonspecific factors as temperature and pH in the evolution of viral growth—as well *in vivo* as *in vitro*—(Lwoff, 1959) is a very convincing argument for the view that "because it is simple it is not necessarily wrong."

IV. Infectious Heredity; Proviruses

The potentiality to produce a virus may be carried as an inherited character. Only in the case of phage-bacteria systems has the nature of the permanent association between host cell (lysogenic bacterium) and provirus (prophage) been really cleared up in a satisfactory way (Lwoff, 1953; Jacob and Wollman, 1957). For this reason this section will be confined essentially to lysogeny and to some more or less related fields in bacterial genetics.

A. *Lysogeny*

Many bacteriophages (*temperate* phages) can infect sensitive cells in either of two ways. One leads to the lytic response (see Section III, B), the other (*lysogenization*) does not. The infected cells which do not lyse, at first glance seem to be unchanged and continue indefinitely to divide. At least part of the cells arising from their first divisions, however, have acquired and transmit to their progeny in an absolutely stable way some properties which characterize lysogenic bacteria: (1) They are immune to infection by the homologous phage and most of its mutants. This immunity does not, usually, prevent the penetration of phage genetic material, which behaves as an inert particle and is diluted out in the course of bacterial divisions (Bertani, 1953; Jacob, 1954). (2) They carry the potentiality of producing phage identical to the strain which was used for infection. In a lysogenic culture, phage particles are usually present. It was shown that they arise from the occasional lysis of a cell, which releases the same number of phage particles as a sensitive bacterium infected with the same phage (Lwoff and Gutmann, 1950). This event occurs with a rather low frequency (10^{-2} to 10^{-5} per bacterium per generation time), depending on the lysogenic strain and the cultural conditions. (3) Many lysogenic strains can be induced to release phage by several agents. The most commonly used agent is UV irradiation, which brings about lysis, with production of phages by a large fraction of the bacterial culture (Lwoff *et al.*, 1950).

Naturally occurring lysogenic strains are very common, and their properties are identical with those of strains made lysogenic by infection with the homologous phage. Lysogeny is exceedingly stable, and isolation of nonlysogenic bacteria from a lysogenic strain can be brought about only by the use of such treatments as strong UV irradiation, which leaves very few surviving cells. No infectious particles can be found by disrupting lysogenic bacteria (Burnet and McKie, 1929), nor can substances serologically related to phage be detected in extracts from lysogenic bacteria (Miller and Goebel, 1954).

It is clear that each cell in a lysogenic strain carries as a noninfectious unit all the information necessary to produce a given type of phage. This unit is known as *prophage* (Lwoff and Gutmann, 1950). What is the relationship between a prophage and the genetic material of the bacterium? In crosses between F^+ and F^- *Escherichia coli* strains the lysogenic character behaves essentially as a bacterial genetic marker, with a precise location on the bacterial chromosome. Lederberg and Lederberg (1953) and Wollman (1953) showed that the inducible prophage λ of *E. coli* K12 is closely linked to a cluster of markers involved in galactose fermentation, whereas Frédéricq (1953a) was able to show that a noninducible prophage of *E. coli* Lisbonne is linked to the xylose marker. When lysogenic strains were "cured" from their prophage and then reinfected and again made lysogenic for the same phage, the prophage was found to be located at the same chromosomal site. Crossing two strains lysogenic for different mutants of the same phage (λ) enabled Appleyard (1954) to show that the determinants of the lysogenic character not only control the ability of the bacterium to produce phage, but also the hereditary traits of the phage produced. The genetic determinant of lysogeny, which can be located on the bacterial chromosome, must thus be identical to the prophage itself. This conclusion was confirmed and strengthened by Jacob (1955), using transduction (see Section IV, E) of the lysogenic or nonlysogenic character, and by Jacob and Wollman (1956), using crosses between Hfr and F^- cells. It appeared from previous work with F^+ and F^- crosses that the behavior of lysogeny in reciprocal crosses between lysogenic and nonlysogenic bacteria is asymetrical: the lysogenic character of the F^+ parent is found among the recombinants only if the F^- cell was lysogenic. Crosses between Hfr and F^- strains, which give a high frequency of recombination for some markers, have shown that the abnormalities are due to the fact that the prophage of a donor lysogenic strain is induced immediately as it penetrates into a nonlysogenic F^- cell. On the other hand, if the receptor cell is lysogenic for the phage carried by the Hfr cell, its immunity prevents induction of the prophage. Since this *zygotic induction* is followed by lysis of the F^- cell, abnormal segregation patterns for lysogeny and the linked markers take place when the donor parent is lysogenic and the F^- parent is not.

number of temperate phages were isolated from *E. coli* by Jacob and Wollman. All the UV-inducible prophages were found to be localized in the same region, near the galactose markers, whereas none of the noninducible prophages were found in this region of the bacterial chromosome. Moreover only the UV-inducible prophages exhibit the phenomenon of zygotic induction. A number of UV-inducible phages were observed to be serologically related to each other and to yield recombinants. They are homologous, except for a short region of the linkage map that controls the ability to lysogenize, the specific immunity, and the location on the bacterial chromosome (Kaiser and Jacob, 1957).

It might be thought that the immunity is due to some kind of steric hindrance, the region of attachment in the bacterial genome—supposedly necessary even for the lytic response—being already occupied. That this is probably not true is indicated by the following results. Bertani (1955) found that prophage P2 of *E. coli* has at least three different possible locations, with a strong preference for one of them. Even when prophage P2 is localized in the "second" position, leaving the most probable region of attachment free, the lysogenic bacterium is immune. Recent evidence strongly suggests that immunity in lysogenic bacteria is ensured by a cytoplasmic specific repressor, whose synthesis is under phage genetic control (Jacob and Campbell, 1959).

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B. Defective Lysogenic Strains

These strains are frequently found among the survivors of heavy UV irradiation. Although most of them lyse after UV induction, only a very small fraction of the lysed cells produce phages (and this production was shown to be due to back-mutation to the wild type). The lysogenic character of these strains can be demonstrated by their immunity against homologous phage. As Jacob and Wollman (1957) have remarked: "If it should happen that defective bacteria are not able to adsorb the homologous phage, their immunity pattern cannot be tested. Obviously, if one were not aware of the origin of such bacteria, their lysogenic character would hardly be recognized."

Superinfection of defective lysogenic strains with normal homologous phage, after induction, enables defective phage growth and maturation. Defective phage particles can be obtained, which may in turn be used to produce defective lysogenic strains. It is possible to map the defective mutation on the linkage group of the prophage, and, on the other hand, to find out what stage of phage development is blocked. This line of research seems to be one of the most promising with respect to the study of phage morphogenesis and its genetic determination.

C. Colicinogeny

Some strains of bacteria produce *colicins* (Frédéricq, 1953b, 1954, 1957; Frédéricq and Betz-Barbeau, 1952), diffusible substances, apparently proteins, which have bactericidal action on other related strains. Colicins act on sensitive strains through specific receptors controlled by genetic factors. Some colicins have the same receptors as some phages, and the ability to adsorb such a colicin is therefore under the control of the same genetic locus as the ability to adsorb the corresponding phage. Unlike phages, colicins are not transmissible agents. The hereditary ability to produce a colicin (colicinogeny), however, has profound similarities with lysogeny. A strain that produces a given colicin is immune toward the action of this colicin. This immunity can be overcome by UV induction, and the cells then lyse with colicin production. The genetic determinants of colicinogeny, like those of lysogeny, may exist, when present, in two alternative states, either as autonomous units replicating independently of the bacterial chromosome, or as integrated units attached to the bacterial chromosome with which they replicate (Alfoldi *et al.*, 1958). Similar situations observed with other genetic elements (e.g., the sex factor F^+) led to the definition of a new class of genetic elements, for which the term *episomes* was proposed (Jacob and Wollman, 1958).

D. Conversion

Various properties of bacterial strains, including production of toxin by *Corynebacterium diphtheriae* (Freeman, 1951; Barksdale and Pappenheimer, 1954) and of some antigens by *Salmonella* (Uetake *et al.*, 1955) were found to be correlated with the lysogenic state for a given phage. The acquisition of such a property by lysogenization is called *lysogenic conversion.* Recent work, however, has shown that conversion is rather the result of the expression of the phage genome in the bacterial host, whether as prophage or as vegetative phage (Uetake *et al.*, 1958; Zinder, 1958).

E. Transduction, Transformation

Lysed donor bacterial cells can provide short fragments of genetic material for receptor cells. This transfer can be mediated by free DNA from the donor cell (*bacterial transformation*) (Avery *et al.*, 1944). As there is no device for injection of these naked DNA molecules, success depends on a special physiological state of the recipient cell, "competence" (McCarty *et al.*, 1946; Hotchkiss, 1954; Ephrussi-Taylor, 1955; Thomas, 1955).

Similar genetic transfers can also be observed when phage obtained from the lysis of a bacterial strain is used to infect another strain. Among the survivors, a small fraction is found to have acquired one or more closely linked, characters from the donor strain (*transduction;* see: Zinder, 1955). It is believed that short pieces of genetic material from the lysed donor cell may occasionally be included inside the membrane of a phage particle and injected into the recipient cell at the same time as phage genetic material. Transduced material is usually believed to be transferred as DNA molecules, by analogy with bacterial transformations. The insensitivity toward DNAase would be due to the protection by the phage coat. This type of transduction ("generalized transduction") can be used for the transfer of any bacterial marker—including lysogeny for another phage—and has proved to be a powerful tool for analysis of the fine structure of bacterial genes (Demerec *et al.*, 1956). Not all phages are able to transduce bacterial genetic material: of many independently isolated *E. coli* temperate phages, Jacob found only one transducing phage.

A different type of transduction was discovered by Morse (1954). Whereas *E. coli* infected with λ, or related ("lambdoid") phages, do not yield transducing phage, induced lysogenic strains yield lysates which are able to transduce, with a frequency of about 10^{-6} per phage particle, the galactose makers (which are closely linked to prophage λ). Most of the transduced cells yield clones whose UV induction gives lysates with high transduction frequency, approaching one per phage particle (Morse *et al.*, 1956a, b). It was found (Campbell, 1957; Arber *et al.*, 1957) that these strains are doubly lysogenic for a normal, nontransducing phage and for a defective transducing phage, which seems to have a region of its genome substituted by the galactose region of the bacterial genome.[7] The most interesting feature of this system is probably that a typically bacterial genetic fragment—the region controlling galactose fermentation—can be induced to adopt the unbalanced rate of multiplication of the vegetative phage.

Lysogeny shows in a remarkably clear way, how intimate can be the genetic relaionship between a virus and its host cell. There is indeed increasing evidence for some homology between part of the phage genome and part of the bacterial genome, even for some virulent phages (see, for instance, Weigle, 1953; Fraser, in Hershey *et al.*, 1954; Fraser, 1957; Stent, 1958). In typical lysogenic bacteria, the phage genome behaves much as part of the bacterial genome, whose rhythm of multiplication it adopts. In some cases the distinction between viral and host genetic material may even tend to vanish, at least operationally.

The idea that mechanisms similar to lysogeny could account for cases of latent viral infections or cancers in plants or animals has been discussed by Lwoff (1953), Jacob (1954), and Jacob and Wollman (1957). An interesting situation has recently been described by Cieciura *et al.* (1957). Animal cell cultures started with survivors of a massive infection with Newcastle disease virus were still immune after two years' transfers without virus. When plated on a layer of X-irradiated HeLa cells, each resistent cell behaves as an infective center. The behavior of a well-known tumour virus, the Rous Sarcoma virus (RSV), was recently analysed by Rubin and Temin (1958). Although there are striking similarities between temperate phages and this tumour virus, there are also important differences: in addition to the fact that RSV probably contains only RNA, Rous Sarcoma cells can make mature virus and yet continue to divide. In addition to such typically viral infections, some borderline cases are also known in higher organisms: especially well studied are the abovementioned "kappa" factor in *Paramecium* (see: Beale, 1954) and the "génoïde" in *Drosophila* (see: L'Héritier, 1957).

V. Discussion

Mature virus is found as metabolically inert particles that often tend to crystallize (either inside the host cells, or *in vitro* after purification). It would be an oversimplification, however, to call these particles "nucleoprotein molecules." The upper limits in size and chemical complexity of what may be called a molecule are, admittedly, arbitrary. But the association of, for instance, phage proteins and phage DNA is really very loose. It is apparently more similar to the relationship between a bottle and its contents than to chemical bonds in the usual meaning; moreover, the protein shell itself is differentiated morphologically and serologically and it involves several protein molecular species.

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[7] It would perhaps be worth while to test whether such an abnormal prophage can attach in two different ways to the bacterial genome: either through its normal attachment region, at the specific prophage locus, or through its galactose region, at the galactose loci of the bacterial chromosome.

The complexity of viral particles cannot be compared to that of living cells either. These particles lack enzymatic energy-yielding and synthesizing systems. Their only enzymatic activity is apparently concerned with penetration into the host cell. Moreover, most viruses, perhaps all, contain only one kind of nucleic acid, either DNA or RNA. The biochemical complexity of viruses—or, more precisely, of their genetic moiety—may probably better be compared, in the hierarchy of living systems, to that of cell constituents. DNA-containing viruses are similar to nuclear genetic segments, whereas RNA-containing viruses might be considered as being homologous to the normal ribonucleoprotein granules.

Whether only part of the viral components penetrate inside the host cell is known for certain only in the case of bacteriophages. As pointed out above, however, there is evidence for some kind of dissociation of the viral constituents (eclipse period, sensitivity toward nucleases) in the case of animal and plant viruses as well. Even if the whole infectious particle does penetrate, it could still be possible to distinguish between genetic and nongenetic material by studying, as Hershey did for phages, the transfer of labeled components from parent to progeny virus.

Viral genetic material provides the cell with the complement of information that is needed for the synthesis of more viral substances. The nature of this information may be tentatively described as follows: (a) The *whole* viral information is involved in the synthesis of more "autoreproducible" material endowed with this information (DNA or RNA according to the class of virus). (b) *Part* of this information imposes a specificity on the viral proteins. (c) Macromolecules that were not synthesized by the uninfected cell and that will never be included into mature viral particles appear after infection. It may be presumed that part of these molecules are synthesized under viral genetic control. There are, for instance, enzymes which enable the infected cell to synthesize new building blocks needed for viral growth.

How this information is used, however, depends on a number of factors. We will deal first with the fate of alien genetic material—*whether* *viral or not*—when introduced into a host cell, taking as an example some of the possible responses of alien DNA introduced into a bacterial cell:

(1) The alien DNA remains undegraded, but it is not multiplied. This *abortive* response is found when a lysogenic bacterium is infected with a homologous phage; apparently in abortive transduction (in this case the alien genetic fragment is detected by its phenotypic expression); probably when bacteria that are competent for transformation are provided with DNA from an unrelated species, or when transformation fails.

(2) The alien DNA is integrated into the bacterial genome. This *reductive* response is found in transformation and transduction and, probably in a somewhat different way, in lysogenization.

(3) The alien DNA is rapidly multiplied (*vegetative* response), as in typical productive infection.

Although far from being understood, the determination of the response of extra genetic material is known to depend on physiological conditions of the cell as well as on genetic factors. Reductive and vegetative responses apparently imply some homology between part of the viral genetic material and a region of the host genetic material, allowing pairing (except presumably for such exceedingly virulent phages as the T-even and T5 of *E. coli*); for a discussion of this point, see Stent (1958). As concerns the ability to undergo vegetative multiplication, it may be supposed that *some* genes "distinguish the phage from being merely a piece of bacterial chromosome and . . . are responsible for conferring the viral character to the entire phage genome" (Stent, 1958). It is important to realize that, except for the above-mentioned part of the viral genome the ability for a DNA segment to be multiplied in the vegetative way is not due to some intrinsic "viral character." For instance, the genetic segment that controls galactose fermentation in *E. coli* is sometimes included in the genetic structure of a "lambdoid" prophage (see Section IV, E). If this phage multiplies vegetatively, the galactose markers apparently follow the same pattern of multiplication as any usual phage gene; this bacterial genetic segment thus behaves as part of the vegetative phage when it is included in the phage linkage group.

The use of the information classified under (b) and (c) above may also be quite variable. As an example, the heterocatalytic function of phage genome will be compared in two instances: productive infection and lysogenic state. Lytic infection is characterized by a fuller use of the information concerning virus biosynthesis. Besides the fact that the genetic material itself is multiplied abundantly; the specific phage proteins are synthesized and allow maturation of a number of viral particles; as noted above, both processes presumably imply synthesis, under phage genetic control, of macromolecules that will not appear in phage particles. In a lysogenic bacterium, in addition to the fact that the viral genetic material is multiplied at a much slower rate, in perfect correlation with the bacterial genome, the information involved in the synthesis of specific phage proteins is apparently not used at all; some substances are nevertheless synthesized under control of phage genetic material (see Section IV, D: Conversion). This is a beautiful example of how the same genetic material may function in entirely different ways, as concerns both its own replication and the fulfillment of its synthetic potentialities. Better understanding of the mechanisms involved in this control would presumably help in finding models for the role of genetic material in the differentiation of higher organisms.

Our previous examples concern DNA-containing viruses, which behave much as groups of nuclear genes. RNA-containing viruses interfere at another level with cell metabolism; they are chemically related to cytoplasmic ribonucleoprotein particles. The genetic control of protein syntheses is probably indirect, and ribonucleoprotein particles are widely believed to perform these syntheses as cytoplasmic "delegates" of the nuclear genes. In contrast with "normal" cytoplasmic particles, however, RNA-containing viruses have probably no nuclear equivalent containing the same information,[8] if so, their RNA moiety is apparently the only carrier of the viral information, thus the viral genetic material.

As reported above, there is evidence that DNA-containing viruses such as phages have some homology with part of the host-cell genome. This factor might be a fundamental one (though obviously not the only one) in limiting host range.[9] One may wonder what is—if there is any—the equivalent of this genetic homology in the case of RNA-containing viruses. In some instances, at least (as in the case of those viruses which grow indifferently in insect and in plant cells) their requirements look rather unspecific.

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[8] It seems, for instance, difficult to believe that those RNA viruses which are able to grow in both insect and plant cells find the nuclear equivalent of their information in such widely different cells.

[9] When a phage grows on a bacterial strain, but not on a closely related mutant, the resistance of the mutant may be presumed to be due usually to a rather secondary factor, and it has indeed been overcome in some instances: bacterial mutants which do not adsorb a phage have been successfully infected, using sexual conjugation with a strain lysogenic for this phage, or using transduction. In contrast, if a phage does not grow on a given bacterial species, in view of genetic lack of homology, it may be guessed that the situation cannot be changed. Some phages grow on either *E. coli*, *Shigella*, or *Salmonella;* these species have at least partial genetic homology, as shown by intergeneric sexual recombination, or transduction.

It seems more and more apparent that the fundamental behavior of viral material has profound similarities with that of cellular components of similar chemical composition. As many viruses are amenable to precise titration and to extensive genetic analysis, it may easily be understood how their study became a most powerful tool in attempts to understand cellular mechanisms in general.

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CHAPTER 2

Outline of the Visible Organization of Bacteria

By C. F. ROBINOW

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I. Introduction

Bacteria are ubiquitous free-living cells that are either single or arranged in packets or chains. The parts of the organization of bacteria that can be made visible with light are simple and do not resemble the parts of plant and animal cells. At the molecular level the organization of bacteria is exceedingly complicated, and their metabolism often has striking effects on the composition of their environment. Bacteria first became notorious some eighty years ago as the cause of diseases of animals and man. It was later seen that they also cause disease in plants. More fruitful is the contribution of bacteria to the maintenance of the fertility of the soil and their ability—exploited on a large scale by chemical industries—to synthesize compounds useful to man. In more recent decades experiments with bacteria have provided important information on the synthesis and mode of action of enzymes. The study of bacteria has also contributed much to our knowledge of the life of certain viruses and has widened the horizons of genetics. It cannot be said that a knowledge of bacterial anatomy has been important in the development of any of these fields, but the structure of bacteria will always be of interest to botanists, protozoologists, and others who are concerned with the organization of cells in general. Presumably for this reason a chapter on bacteria has been included in the present treatise. The account that follows is no more than a primer of some areas of bacterial cytology supported by a short list of selected references. It has seemed permissible not to go beyond this because of the simultaneous publication of a comprehensive treatise, "The Bacteria," in which all aspects of the life of these organisms are thoroughly dealt with (Gunsalus and Stanier, 1960). There, too, will be found what is most obviously missing from the present chapter, namely a detailed account of the *chemical needs and capabilities* of bacteria. For a synthesis of morphological and chemical information on bacteria the reader is referred to a recent review of the "Biochemical Cytology of Microorganisms" by P. Mitchell (1959).

II. General Features of Bacteria

The dimensions of bacteria are of the order of a micron, but some are much smaller than this. Relatively large bacteria are found in cow dung (Peshkoff, 1940; Pringsheim and Robinow, 1947), in the cecum of guinea pigs (Robinow, 1957) and other small rodents, and in the gut of tadpoles (Delaporte, 1956). The forms of bacteria are variants of a few basic shapes. They are either spheres (Fig. 1) or rods. The latter may be straight branched, curved, or twisted into a shallow helix (Figs. 2, 3).

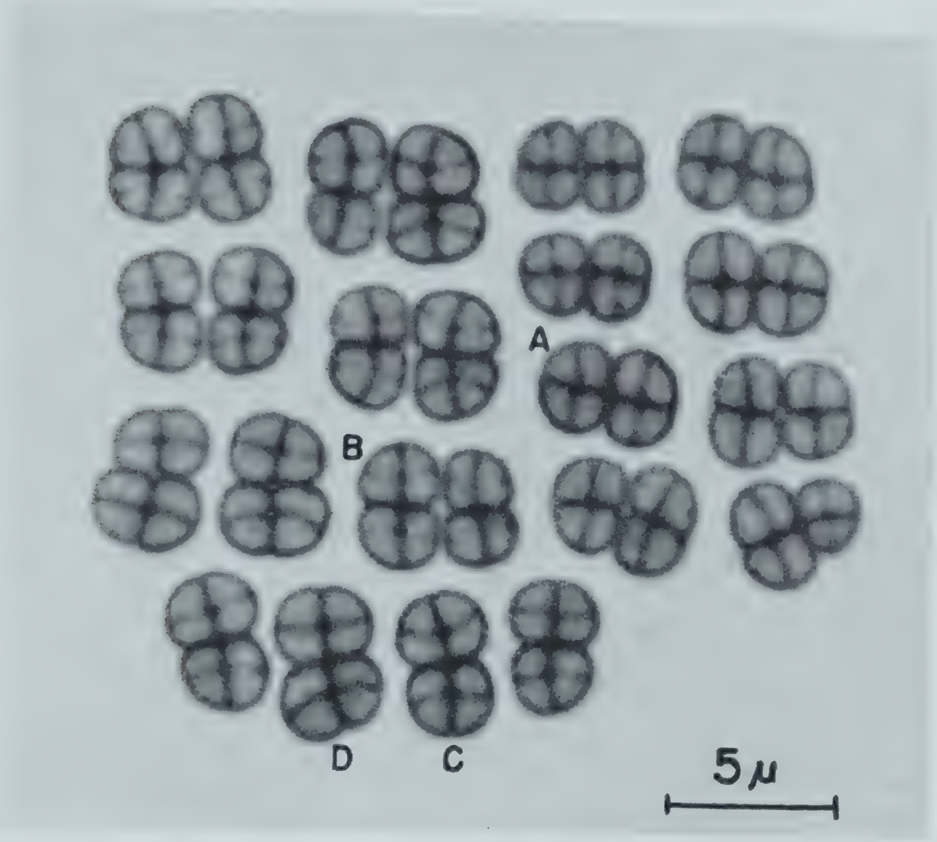

FIG. 1. A colony of rounded, potentially spherical bacteria which tend to form tetrads and octets. Letters indicate successive stages in the transformation of a tetrad (A) into an octet (D). Note the beginnings of new transverse walls in B. Hale's phosphotungstic acid-methyl green stain.

Bacteria of uncommon habit not fitting either of these descriptions are *Caulobacter* (Houwink, 1955) and *Rhodomicrobium vannielii* (Duchow and Douglas, 1949; Murray and Douglas, 1950). The former is a curved rod or vibrio which is able to attach itself with a stalk to the substrate, even to other bacteria. It has a flagellum at the opposite end. *Rhodomicrobium* is a photosynthetic anaerobe with plump spindle-shaped cells which multiply by pushing out a relatively long hollow tube from their cell wall having a lumen less than 0.2 μ in diameter. Protoplasm flows down this tube and forms a bud at the free end which gradually enlarges and in turn pushes out a fresh tube. Tubes may branch at various angles to each other and give rise to flowerlike sprays of budding cells.

Bacteria are essentially single cells, but several of them may be joined together. The unit of movement in a sample of swimming bacteria, "the bacterium" of the unsuspecting first glance, can often be shown to be subdivided (Bisset, 1957), but there is no division of labor between the components of a many-celled bacterium.

Some bacteria are fast swimmers, propelling themselves, it is widely

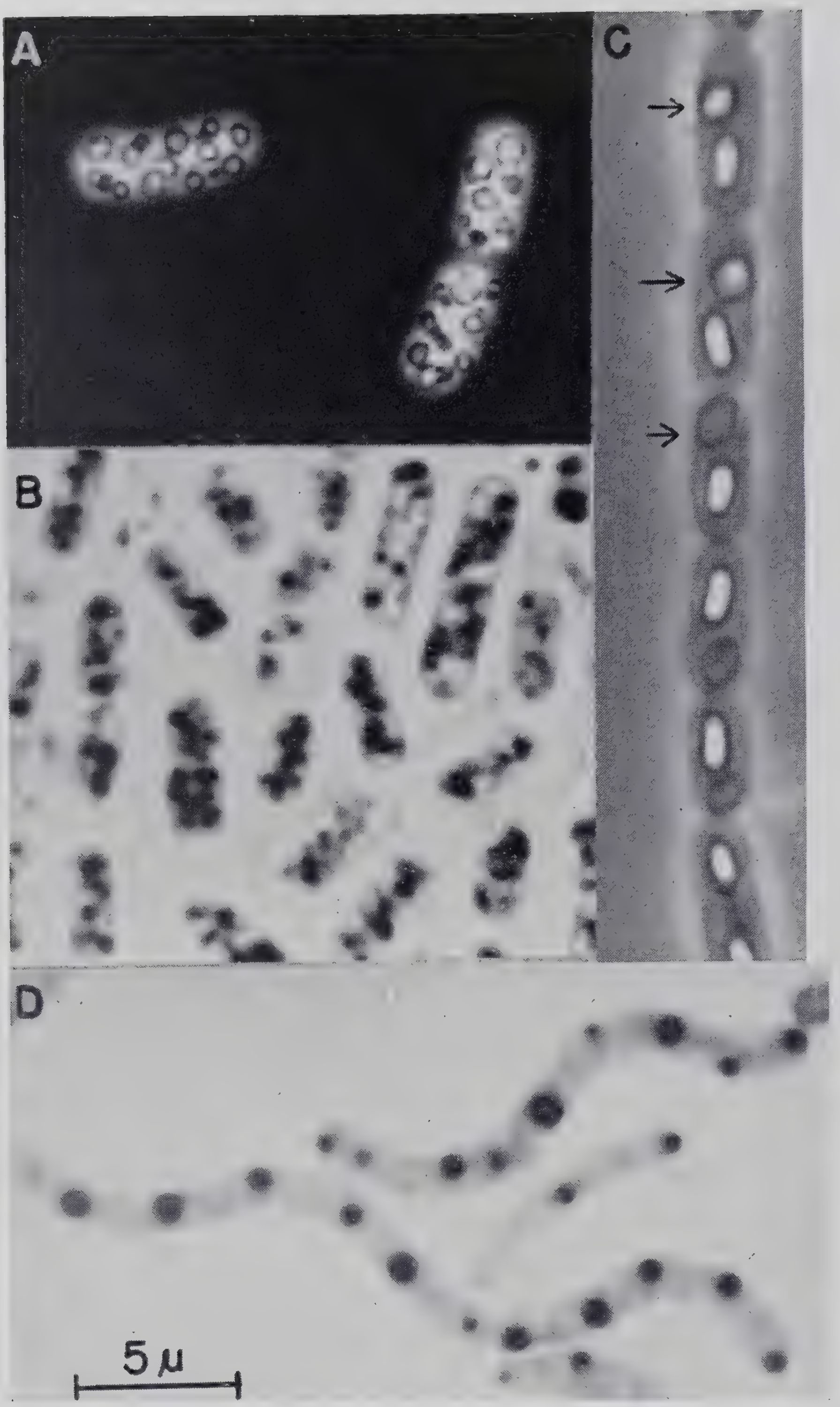

FIG. 2. Examples of different kinds of substances which bacteria store in their cytoplasm: A, sulfur in *Chromatium okenii*; B, lipid (hydroxybutyrate) in *Bacillus megaterium* stained with Sudan black B; C, crystals of protein (arrows) together with spores, one of each in each cell, in *B. cereus* var. *alesti;* D, metachromatic (polyphosphate) grains in *Spirillum volutans* from cow dung. Photograph C by courtesy of Dr. P. C. Fitz-James.

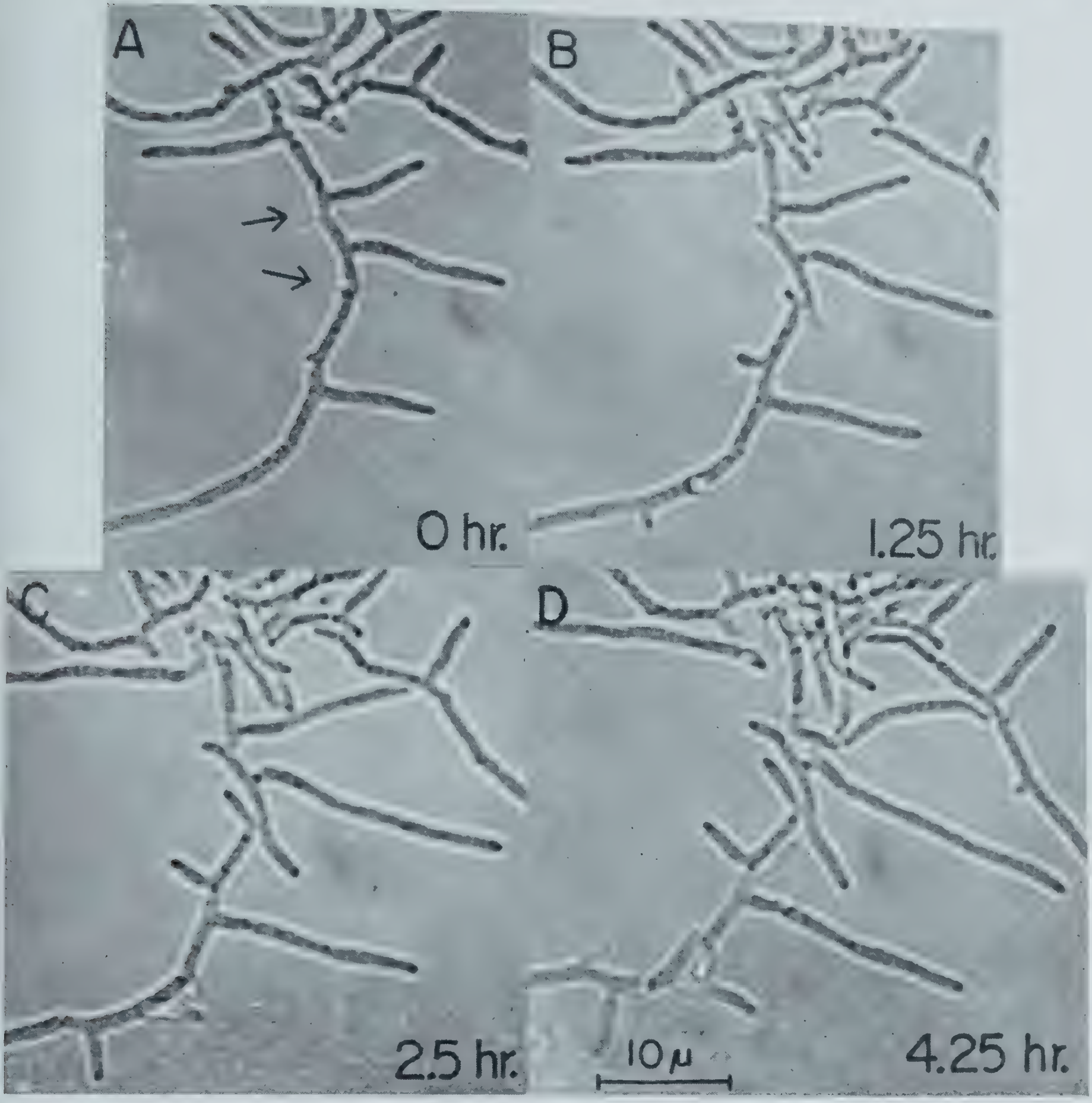

FIG. 3. Four stages in the growth of a branching bacterium (*Nocardia* sp.). A short length of "mycelium" between the arrows in A breaks away and becomes an independent twig in the remaining pictures.

but not universally believed, by the action of flexible, exceedingly fine, hairlike appendages. Others cannot swim but are able to creep and glide on surfaces and interfaces.

The body of bacteria is encased in a tough membrane or *cell wall* which in many species seems to be rigid but is flexible in others. The protoplasm of bacteria is relatively dense and during rapid growth may appear uniformly transparent and featureless. At other times it may contain a few or very many *inclusions* of lipid, granulose (iogen), sulfur, polyphosphate, protein crystals, and other nonliving materials (Fig. 2).

The protoplasm of bacteria can be divided into dense ground cytoplasm and variously shaped masses of lesser density which give a positive Feulgen reaction and are generally regarded as being akin to nuclei. Often, especially during rapid growth, there are well-defined concentrations of this material, which are spoken of as *"nucleoids"* or *"chromatin bodies"* or, loosely, as nuclei. In some instances, e.g., in the gliding bacteria, the chromatin is so evenly distributed throughout the cytoplasm that it is not readily revealed by ordinary microscopy.

Bacteria usually multiply by splitting in two or by pushing out branches which later break away (Fig. 3). An alternative mode of multiplication, the formation of small round buds or "gonidia" has been described of several species and is fairly well established for the genus *Spirillum* (Williams and Rittenberg, 1956). The simple round of growth and division is interrupted, in some species, by the formation of resting cells or *spores* which arise within the cytoplasm of the vegetative form but differ from the mother organism both in structure and in chemical composition. Spores are endowed with remarkable powers of resistance to drying in air and to heat and can remain alive for a long time without detectable metabolism, 114 years in one well-documented instance (Wilson and Shipp, 1938). Until recently bacteria were thought to lack sexuality, but it has now been demonstrated that cells of several strains of several species are capable of transferring, during brief contact, some of their genic material to their fellows and that the genetic constitution of the progeny from such zygotes differs from that of their parents.

The principal features of bacteria will now be briefly discussed and illustrated with representative examples.

III. The Cell Wall

A. *General Observations*

Bacteria owe their shape to the wall which encases their protoplasm. Little more refractile than water and with little affinity for stains, the cell wall is hard to distinguish from the surface of the dense, readily stainable cytoplasm that is everywhere in close contact with it. Before the cell wall can be seen, the cytoplasm has to be pulled away from it. This can be done in several different ways. For example—by crushing bacteria in India ink (Weibull, 1955a). The spaces vacated by the disrupted and dissolved protoplasts fill with ink and there remains only a white, chambered, ink-impenetrable skeleton, i.e., the cell wall. A useful degree of retraction of the cytoplasm from the cell wall is caused by hypertonic salt solutions. An effect of this kind is seen in living spirilla and gram-negative rods when they are examined in 0.2 M NaCl *or* 0.2 M

KNO_3 (Fischer, 1895). Much higher concentrations of salt are required to produce similar effects in gram-positive bacteria (Knaysi, 1930). This will be further discussed under Plasmolysis, Section IX, A.

In stained preparations of killed bacteria the cell wall is again most clearly visible as a separate structure if the protoplast is pulled away from it before fixation although instructive preparations have been obtained by extruding the protoplasts of fixed and stained bacteria out of their cell wall sleeves through pressure exerted with a coverslip forceps (Murray and Robinow, 1952). Fischer (1895) found that bacteria could be preserved with their cytoplasm in states of contraction corresponding to the salt effects described above if they were allowed to dry in a drop of 0.25–0.5% NaCl or 0.5–1% KNO_3 and then stained with an alcoholic, not a watery, dye solution. The method has recently given good results with *B. megaterium* (Robinow and Murray, 1953). In this latter species and other *Bacillus* cells a general, extensive, retraction of the cytoplasm from the cell wall is obtained by first drying the bacilli for a few minutes on glass and then immersing the dried film in Bouin's fixative (saturated watery solution of picric acid, formalin, acetic acid, 15:5:1). Drying alone usually does not suffice to pull the cytoplasm away from its anchorage at the cell wall, but it somehow weakens the bond between the surface of the cytoplasm and the wall to such an extent that the cytoplasm can contract unhindered when the dried bacilli are later immersed in the Bouin reagent (Robinow and Murray, 1953). This morphologically useful effect is greatly enhanced if drying is preceded by exposure of the living bacteria to vapors of ether or chloroform (Fig. 4) (Robinow and Murray, 1953). This treatment: ether, followed by drying, followed by fixation, has the further advantage that it somehow increases the thickness of the cell wall and enhances its affinity for stains. It has been suggested that pretreatment with ether vapor may cause the cell wall to relax and to be lifted off from the cytoplasm (Mitchell and Moyle, 1956), but this does not seem to be so. The dimensions of the cell wall tubes in Fig. 4 are the same as those of the intact living bacilli of the same culture in Fig. 6. Clear images of the cell wall have also been obtained in susceptible species by mild digestion with lysozyme in the presence of specific cell wall antibodies (Tomcsik, 1956a, b).

Most of the modern information about the bacterial cell wall has come from the chemical fractionation and the electron microscopy of isolated walls freed from protoplasm. Empty cell wall tubes can be obtained in large amounts by shaking bacteria with fine glass beads (e.g., of 0.2 mm. diameter) (Dawson, 1949). The protoplasm flows out through the breaks caused by the bombardment and the empty husks left behind are cracked and ruptured, but not otherwise deformed. For

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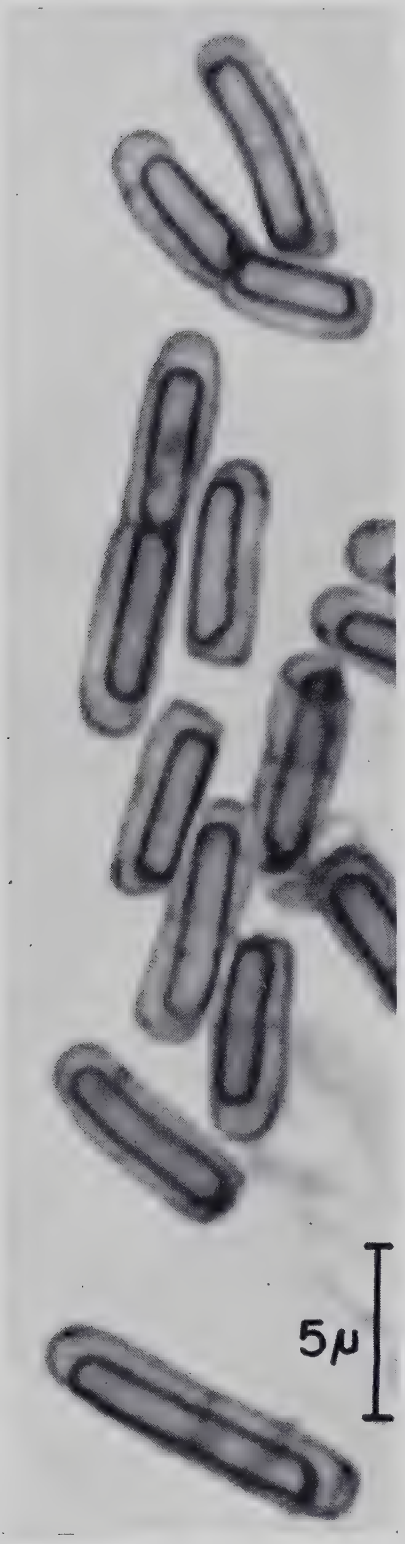

FIG. 4. Retraction of the cell contents (protoplast) from the cell wall induced by exposure to ether vapor and drying on glass before fixation. Explanation in the text. Bouin. Victoria blue B.

chemical studies they may be washed and digested with proteolytic enzymes to remove remains of the protoplasm. Whole cell walls of rod-shaped bacteria appear simple and solid, but profiles of the walls of *B. megaterium* and of *Escherichia coli*, seen in sections, reveal that they consist of several layers of different density. With few exceptions no visible fine structure has been detected in the wall of spherical or rod-shaped bacteria. Yet it is certain that in the wall of rod-shaped organisms there must be considerable orderliness in a plane at right angles to the long axis of the cell because it is usually in such a plane that the cell walls crack when bacteria are bombarded with glass beads or when they break open from within during the lysis that is induced in some of them after exposure to the vapors of ether (Robinow and Murray, 1953).

A rectangular periodic structure has been observed in the cell wall of a rod-shaped bacterium (Labaw and Mosley, 1954a) and in the wall of a spirillum a neat hexagonal array of spherical particles with diameters of 120–140 A. has been demonstrated by Houwink (1953). A similar pattern has been recognized in *Spirillum rubrum* (Salton, 1956) and in *Halobacterium* (Houwink, 1956), and there is a suggestion of it in micrographs of *Selenomonas* (van Iterson, 1956).

A specially orientated fine structure may be expected to exist in the wall of the ubiquitous *B. cereus* var. *mycoides* which grows on the surface of agar in the form of many-stranded curving cables, three out of four of which are invariably deflected counterclockwise relative to the surface of the medium (Murray and Elder, 1949). A similar behavior, based perhaps on an inherent twist of the cell wall, is found in *B. circulans* and *B. alvei*, whose colonies travel over the agar in curved paths, two out of every three of which are deflected counterclockwise (Murray and Elder, 1949).

It is not known in detail how bacteria add to the fabric of their cell walls during growth. In rod-shaped bacteria this is said to happen at special growing points (Bisset, 1951, 1957) at the ends of the cells and around their waists. The protoplasm there is certainly different from that in the rest of the cell. It is relatively dense during life as revealed by phase contrast microscopy, it is more basophilic than the cytoplasm between the chromatin structures (see below), and it remains intact for a long time when variously induced autolysis destroys the rest of the cell (Kellenberger and Huber, 1953, Robinow, 1947).

B. The Cell Wall in Division

Many rod-shaped bacteria divide directly by constriction (Fig. 5). In others constriction is preceded by the inward growth of a gradually

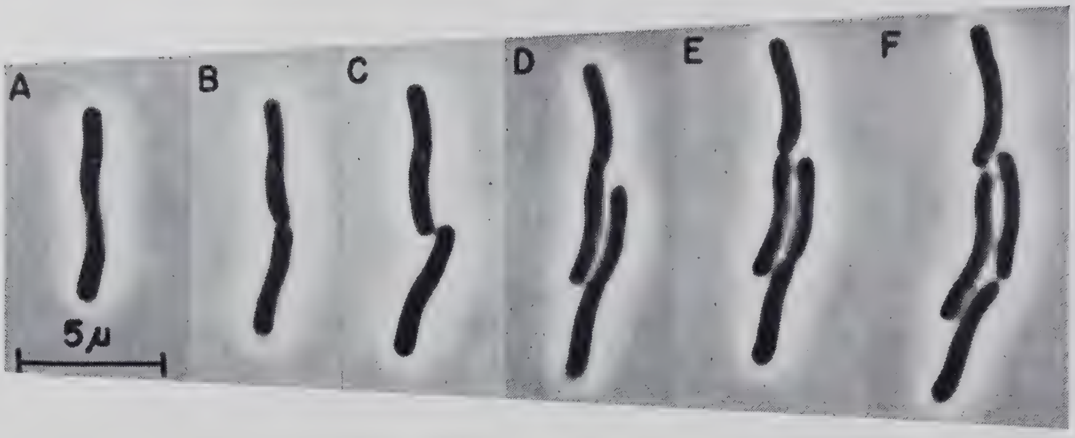

FIG. 5. Successive stages of the division of a spirillum (*Spirillum serpens*). Phase contrast microscopy. Pictures A–E were taken at intervals of 30 minutes, but there are only 7 minutes between E and F.

closing ring-shaped diaphragm (Migula, 1897; Robinow, 1945). The beginning of this process can be discerned as a toothlike thickening of the mid-point of the cell's contours in the bacilli of Fig. 6 and the cocci of Fig. 1 and in the sections of cocci in Fig. 25. Transverse septa in various stages of development are often visible in electron micrographs of sections of bacteria (Chapman and Hillier, 1953; Chapman, 1959). In one coccus in which successive planes of division are at right angles to each other, it has been found, surprisingly, that advancing septum initials turn through a right angle as their growing edges approach to within a certain distance of each other and thereafter continue to grow in opposite directions. They thus proceed to the following division before the previous one has been completed (Murray and Robinow, 1958).

C. Composition of the Cell Wall

Chemical analysis of mechanically isolated cell walls freed from protoplasm has shown that the walls of bacterial cells, unlike those of plants and fungi, do not consist mainly of one relatively simple polymeric material such as cellulose or chitin, but are complicated mixtures of carbohydrates, polypeptides, or even proteins and lipids.*

Detailed reviews of the rapidly growing knowledge of the composition of bacterial cell walls have been published by Salton (1956), Cummins (1956), and Work (1957). The composition of the cell wall is not the same in all kinds of bacteria. According to their response to the staining procedure of Gram, which is important in the diagnostic medical laboratory

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* One bacterium, *Acetobacter xylinum*, can make cellulose, but it does not fashion its wall from this material. Instead, it excretes the building blocks into the culture medium and induces them to polymerize into a feltwork of fine fibrils in which the bacterium gets gradually immured (Aschner and Hestrin, 1946).

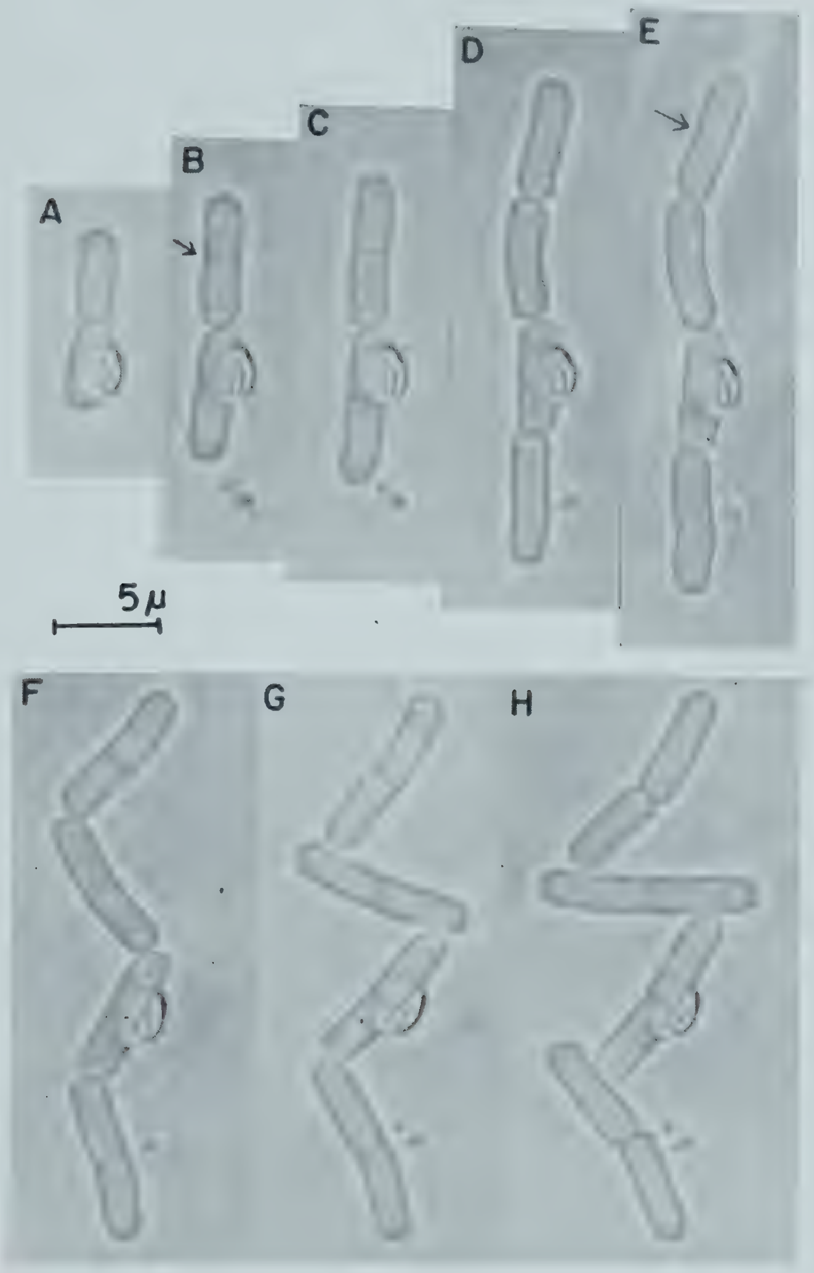

FIG. 6. Time lapse photographs of two successive divisions of a rod-shaped bacterium (*Bacillus megaterium*). The interval between A and H is 1½ hours. Arrows in B and E point to early stages in the formation of transverse walls. The luminous crescent clinging to the lower bacillus in A and which is visible in all the other pictures, is an optical section of the discarded coat of the spore which had given rise to the first bacillus of the chain an hour or so before picture A was taken.

because it is correlated with susceptibility to antibiotics and other important properties, bacteria are classified as being either gram positive or gram negative. In recent years it has been shown that this difference is paralleled by marked differences in the composition of their cell walls. That gram positiveness is not dependent only on the chemical composition of the cell wall is, however, evident from the fact that yeasts are strongly gram positive although their wall differs in composition from that of gram-positive bacteria (Northcote and Horne, 1952). In gram positives, of which the staphylococcus and *B. megaterium* are typical examples, the wall amounts to some 20% of the dry weight of the cells, contains a small number of amino acids, including diaminopimelic acid (Work, 1957) (which is found only in bacteria and blue-green algae), hexosamines, and 1–3% lipid. The wall of gram-negative bacteria, of which the much studied *Escherichia coli* is perhaps the most widely known representative, is thinner, contains more lipid, and is chemically more elaborate than that of gram positives. From the studies of Weidel and his collaborators (Weidel and Primosigh, 1958) it appears that the lipid in the wall of *E. coli,* of which there is about ten times as much as in gram-positive bacteria, is part of two distinct layers. One, extractable with 90% phenol, is made of lipoprotein and amounts to about 80% of the dry weight of the wall. The other layer, which retains its rod shape even after most of the solid content of the wall has been removed with phenol, and which is dissolved by enzymes obtained from the T-group of bacteriophages, consists of the same entities which we have already encountered as the main constituents of the walls of gram-positive bacteria, namely: lipopolysaccharide, diaminopimelic acid, glutamic acid, alanine, glycine, lysine, and hexoseamine. Gram-negative bacteria thus seem to differ from gram-positive ones principally in having the rigid form-giving stratum of their wall covered with (or impregnated by) a heavy layer of lipoprotein. The study of phage adsorption suggests that in many places the deep layer is directly accessible through gaps in the covering stratum.

The finished wall is very stable and can be broken down only by drastic procedures such as many hours of hydrolysis in boiling 2*N* HCl. Remarkable indeed are the capabilities of bacterial protoplasm, watery and chemically very vulnerable, which synthesizes at a fast rate such a complex inert polymer. Analysis of bacterial cell walls and the identification of certain compounds accumulating in bacteria exposed to penicillin have provided a clue to the mode of action of this antibiotic (Park and Strominger, 1957). It seems that the drug interferes with the synthesis of important constituents of the cell wall. Lack of a properly constructed wall leaves the cytoplasm at the mercy of the usually too hypotonic environment, and with no cell wall to take the strain of their high internal osmotic pressure the bacteria disintegrate. This explains why penicillin acts only on *growing* bacteria. Once made, cell walls are invulnerable to its action.

D. Function of the Cell Wall

Bacteria, as Weibull (1953) was the first to show, can be kept alive in the form of so-called gymnoplasts or protoplasts after their cell wall has been completely dissolved, provided the experiment is carried out in the presence of suitable concentrations of substances such as glucose or sodium chloride, which do not readily penetrate the surface of bacterial protoplasm. The function of the stabilizing substances is to balance the internal osmotic pressure of bacterial protoplasts which may range from 5 to 30 atmospheres. To give two examples:

In the original experiments of Weibull the cell wall of *B. megaterium* was dissolved with lysozyme and the resulting protoplasts were stabilized by 0.1–0.2 M sucrose or by 7.5% solution of polyethylene glycol of average molecular weight 3000 to 3700. Very stable protoplasts of *B. subtilis* have been obtained in $M/20$ phosphate buffer at pH 7 containing 0.5 M saccharose and 1 mg./ml. of lysozyme (Wiame *et al.*, 1955). $M/2$ and $M/4$ NaCl were also used with success. Intact cells of both species, as is well known, are able to live and multiply in the presence of concentrations of NaCl ranging from minute amounts of the order of mere impurities to 1 M or higher. A much narrower range of solute concentrations is tolerated by protoplasts. Over this range they behave as osmometers, shrinking or swelling as the concentration is altered, but below a critical concentration (0.1 M for *B. megaterium*) they lyse. Thus it is evident that "in intact cells the mechanical strength of the cell wall prevents the disruption of cells suspended in dilute media or pure water" (Weibull, 1958). The cell wall also appears to be essential for another activity: the synthesis of more cell wall. Up to the present, although protoplasts have been kept alive for a long time and can be induced to grow to ten to twenty times their original volume (Fig. 7) (Fitz-James, 1958) they have not been seen to divide, nor have they made a new wall for themselves.

IV. L-FORMS OF BACTERIA

Protoplasts have properties in common with certain forms of microscopic life which are neither bacterium nor virus, are not cellular, can start fresh growth from particles of the order of size of vaccinia virus, yet can be cultivated outside living cells. These are the pleuropneumonia-like organisms (PPLO), which are named after a contagious disease of cattle caused by one of them. There are parasitic as well as free-living

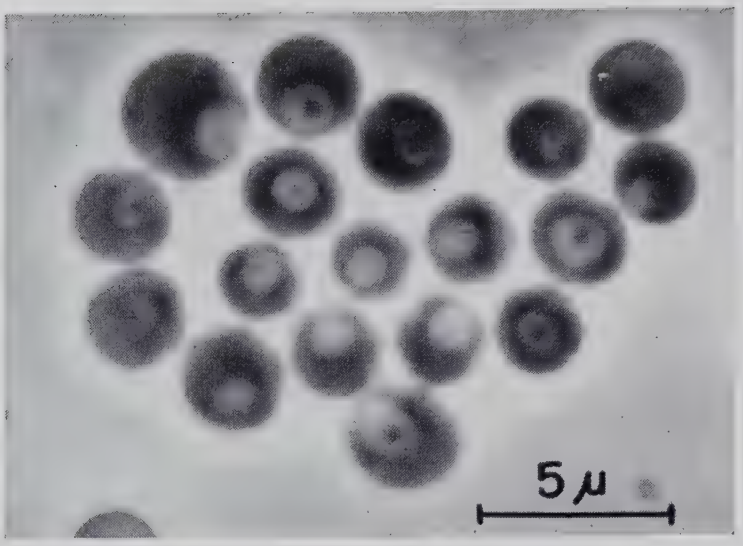

FIG. 7. Living and growing protoplasts of *Bacillus megaterium* maintained in a stabilizing and nourishing medium. The light-gray areas correspond to the chromatin. Reprinted, with permission, from Fitz-James (1958).

varieties. In addition to the naturally occurring forms there exist spontaneous variants of bacteria which adopt the PPLO mode of life (Klieneberger–Nobel, 1947). In this phase they are called L-forms after the Lister Institute in London where the first of the series was isolated and where much excellent work on their biology has been carried out by Klieneberger–Nobel (1954).

Artificial counterparts to these organisms can be prepared in the laboratory. Bacteria prevented by penicillin from forming a normal cell wall can be kept alive and will grow and multiply if they are exposed to the drug in the presence of $0.6\,M$ glucose and suitable nutrients. Colonies of these osmotically fragile elements ("sphaeroplasts") resemble those of the spontaneously arising L-forms (Lederberg, 1957). The body of all these forms of life (the term *cell* seems hardly applicable) is soft and shapeless and either lacks a cell wall altogether or is weakly supported by a chemically defective one. It is still being debated whether the L-forms of bacteria are the same as the naturally occuring PPLO or differ from them in several basic characters. Klieneberger insists on a sharp separation of the two groups (Klieneberger–Nobel, 1956), but the characters which she regards as important, such as the range of sizes of the reproductive units, nutrient requirements, and greater or lesser ease of cultivation, are not more fundamental than those separating species of bacteria belonging to the same or related genera. These matters will remain controversial until the mode of reproduction of PPLO, L-forms, and L-type phases of penicillin-sensitive bacteria has been clearly worked out. Photomicrographs, electron micrographs, and filtration experiments have led some workers to accept minute *intraprotoplasmatic particles* (a kind of gonidium) which are set free at the end of the life cycle as the unit of reproduction (Klieneberger and Smiles, 1942; Tulasne, 1953). Others have found that they can most simply account for the results of filtration by assuming that PPLO and L-forms multiply by forming *buds* of a wide range of sizes down to very minute dimensions (Kellenberger *et al.*, 1956). The proceedings of a recent discussion of all aspects (morphological, biochemical, medical) of this field have been published by the New York Academy of Sciences (Symposium on Pleuropneumonialike Organisms, 1960).

V. CAPSULES (FIG. 8)

Some bacteria have the habit of excreting colloidal materials, polysaccharides, polypeptides, even proteins, which accumulate outside their walls and wrap the cells into a slimy or gummy coat or *capsule* (Tomcsik, 1956a, b). The size of these envelopes varies with the composition of the medium and the general conditions of cultivation (Duguid, 1948). On the whole, capsule formation is favored by an excess of carbohydrates over utilizable compounds of nitrogen. The capsules of the pneumococcus are an important part of the unknown means by which this organism causes disease and have become famous through the demonstration that the ability to secrete them can be transferred from capsulated to noncapsulated cells and their descendants with the aid of a "transforming principle"—identified as deoxyribonucleic acid (DNA)—extracted from the capsulated form (Avery *et al.*, 1944).

The capsules of the pneumococcus and of many other bacteria consist of polysaccharide (Heidelberger, 1956), those of the anthrax bacillus are polymers of *d*-glutamic acid (Ivanovics and Bruckner, 1937; Thorne, 1956), a capsule of protein surrounds the plague bacillus (Amies, 1951), and the capsules of many *Bacillus* species contain both polypeptide and polysaccharide (Tomcsik, 1956a, b). In some strains of *B. megaterium* the capsules are complicated structures in which a diffuse matrix of polypeptide is supported by a scaffolding of polysaccharide (Tomcsik, 1956a, b).

Capsules are usually readily soluble in water, and since they also lack affinity for most stains they are usually demonstrated indirectly. In the most generally applicable method, capsulated bacteria are dispersed in India ink and observed wet under a coverslip (Duguid, 1951). In the capsule of the Lisbonne strain of lysogenic *E. coli*, electron microscopy has revealed randomly arranged striated fibres with periodicities around 230 A. (Labaw and Mosley, 1954b). Layers of fibers, concentric

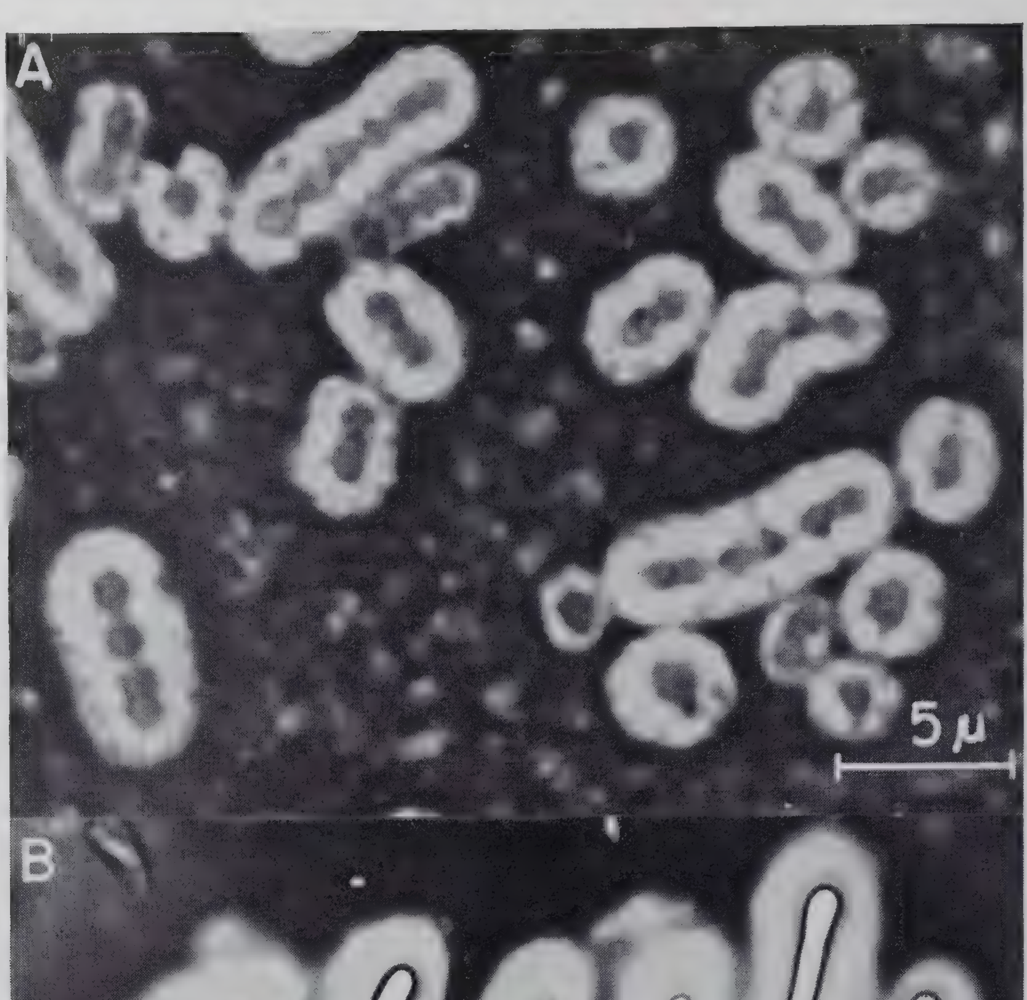

FIG. 8. Capsules of two kinds of bacteria thrown into relief by dark colloids (Congo red in A, nigrosin in B) to which they are impenetrable. A. *Diplococcus pneumoniae* (pneumococcus) in the spinal fluid from a case of meningitis. B. *Clostridium pectinovorum,* anaerobic; 2 days.

in this instance, have also been described in the capsule of *Azotobacter* (Carr, 1958). The organization of capsules can be studied by following in the phase contrast microscope the course of the reaction of capsules with specific antigens. Such studies have been thoroughly and elegantly carried out by Tomcsik and his collaborators (1956a, b).

VI. FLAGELLA (FIGS. 9–12)

Motile bacteria were early distinguished from inanimate objects of similar shape and size because in a fluid medium they swim rapidly back and forth across the field of the microscope. Bacteria which can move through water do so with the aid of one or more tails which on drying usually fall apart into a number of fine hairs or "flagella" (Figs. 9–11) (Pijper, 1957). The flagella that make up the tails may emerge separately from different points of the surface of the bacterium (Fig. 12) or

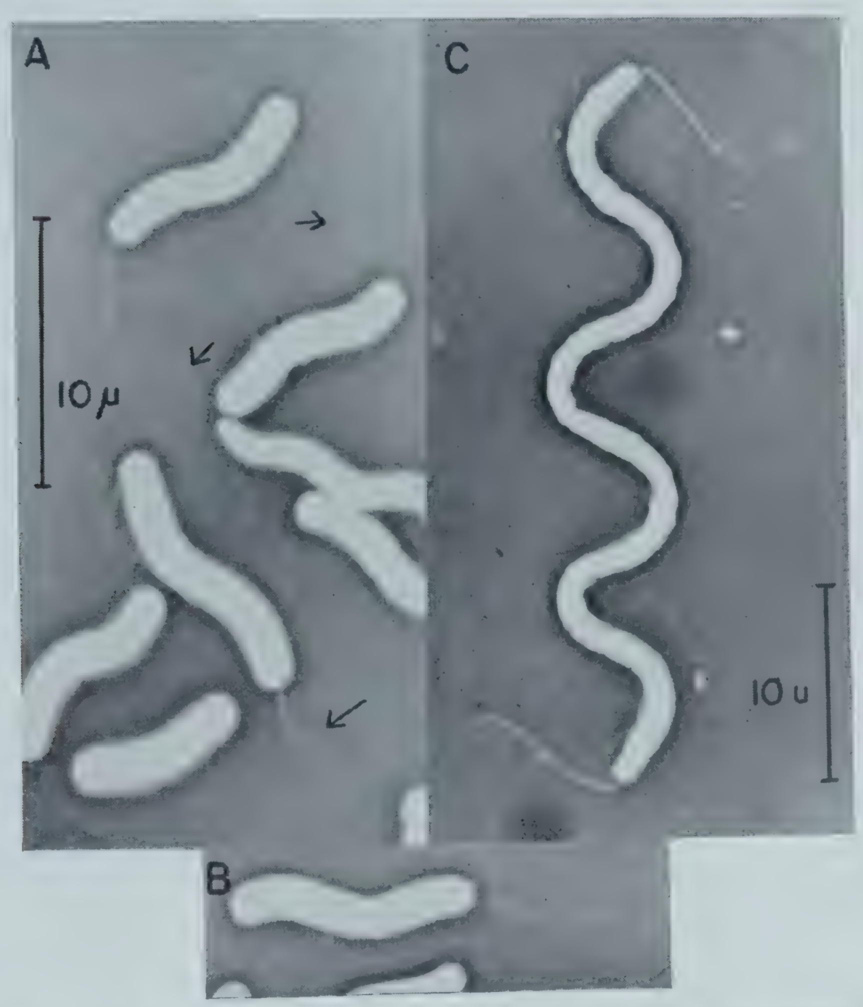

FIG. 9. Two kinds of spirilla preserved in a dried film of nigrosin with their whiplike compound tails still intact. A. *Spirillum serpens*. B. A much larger spirillum from stagnant water.

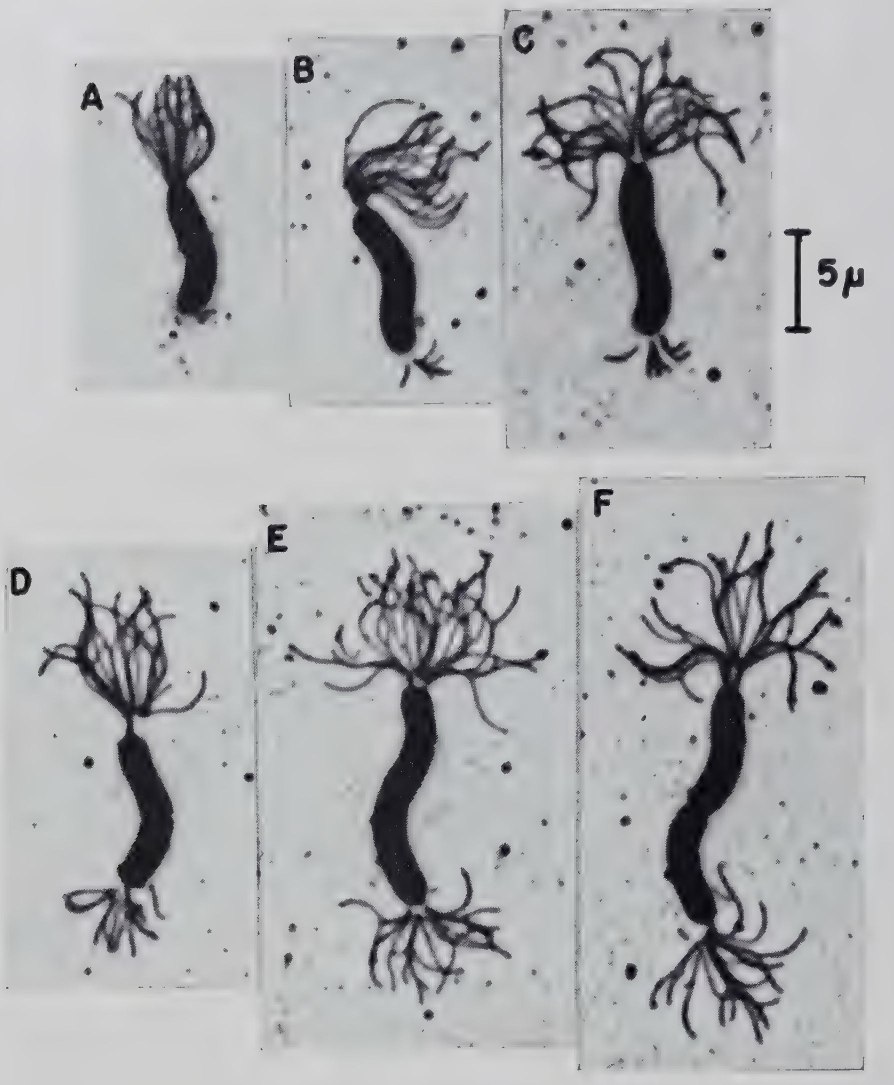

FIG. 10. Fixed and stained spirilla from a randomly growing culture of *Spirillum serpens*. The normally single (but compound) tail has split into many flagella. A. A cell which has just emerged from division and has no flagella at the pole that has been newly formed by constriction (see Fig. 4, illustrating the same strain). B–F are arranged to illustrate the outgrowth of new flagella from that pole of the cell. The spirillum in F, as its slight constriction indicates, is about to divide. The flagella in the new bunch are still somewhat shorter than those of the original tuft at the other end. Some of the photographs closely resemble a set of drawings published by A. Fischer in 1897. Gray's stain. The combined effects of mordant and dye have greatly increased the thickness of the flagella.

all together from one or both poles (Houwink and van Iterson, 1950). A single flagellum forms the tail of *Vibrio metchnikovii* (van Iterson, 1949). Flagella are aggregates of elongated molecules of a protein named "flagellin" (Astbury *et al.*, 1955) having molecular weights of 40,000 or, perhaps, 20,000 (Koffler, 1957). Flagellins are "architecturally similar to the proteins of the keratin-myosin-epidermin-fibrinogen class" but, according to Koffler (1957; Kobayashi *et al.*, 1959), differ from all of them in their

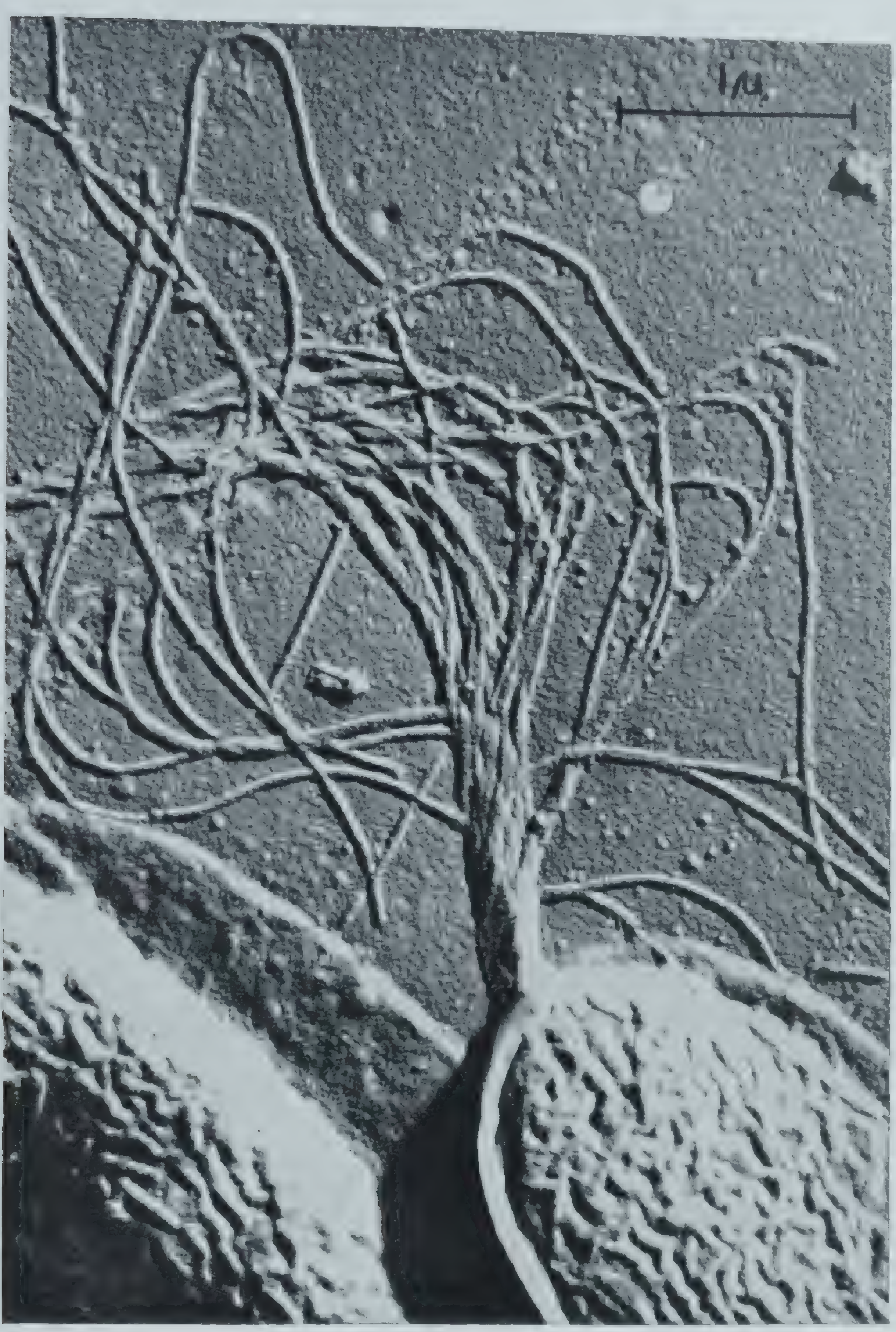

FIG. 11. Electron micrograph of a shadowed carbon replica of *Spirillum serpens*. The compound tail has been fixed in the process of unwinding into its component flagella. Compare with Fig. 10, D. Preparation and micrograph by Dr. P. C. Fitz-James.

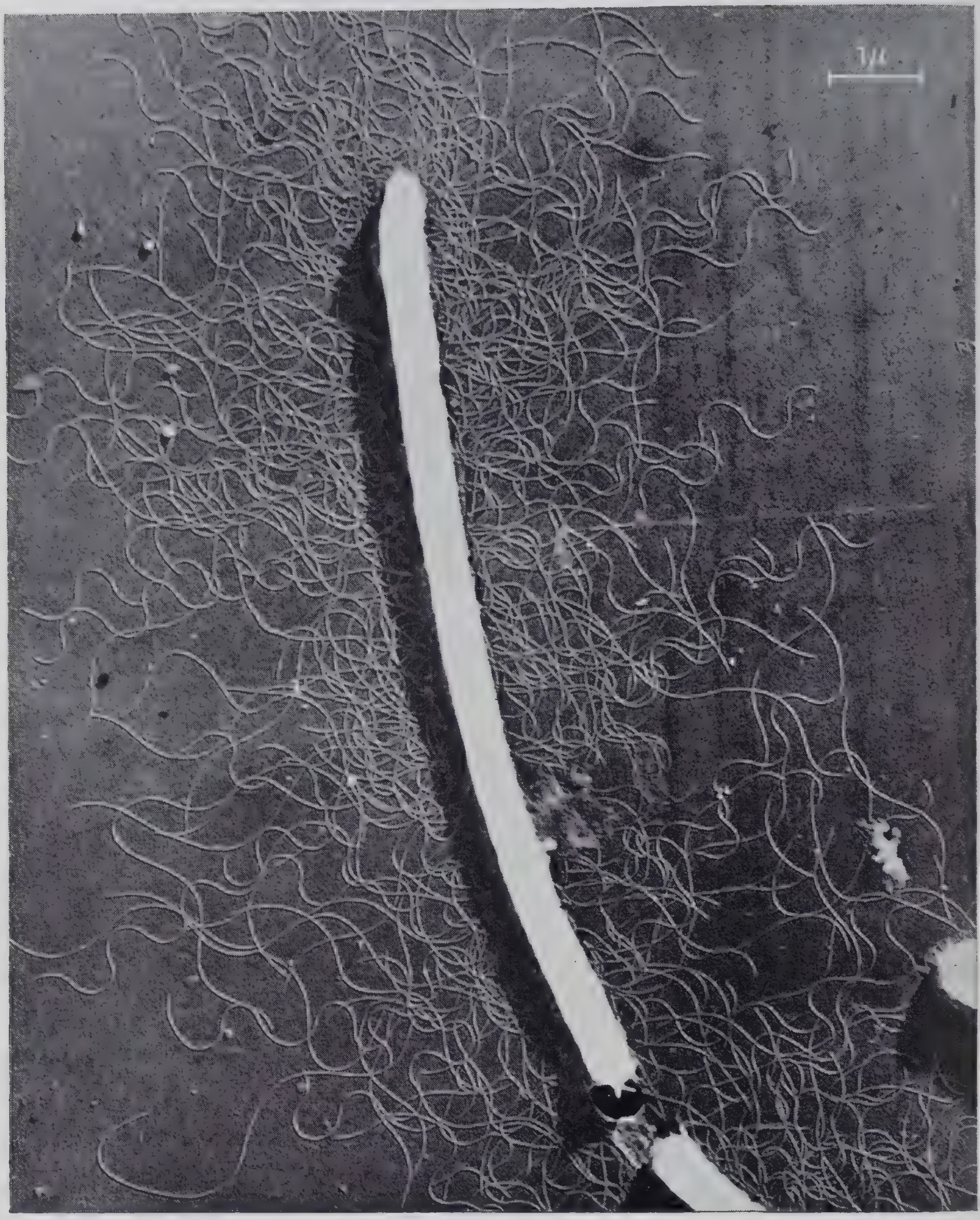

Fig. 12. *Proteus vulgaris* with its mane of long wavy flagella. In life the mane is plaited. From a specimen prepared by James Hillier and C. F. Robinow.

amino acid composition. Flagella are from 3 to 12 μ long, have a uniform thickness of about 120 A., and usually end squarely. A helical fine structure has been detected in some of them (Labaw and Mosley, 1955; de Robertis and Franchi, 1951; Starr and Williams, 1952) and relief staining with phosphotungstate by the method of Brenner and Horne (1959) is said to have shown that flagella are hollow. To make flagella separately visible in transmitted light several methods can be used. They are, for example, first mordanted and then stained. The two operations may also be carried out together (Leifson, 1951). The mordant (tannic acid or potassium aluminum sulfate or both) prepares the flagella for the acceptance of dyes (usually fuchsin) and at the same time causes them to swell. This effect has not yet been exploited by electron microscopists. It has been known since the time of A. Fischer (1895) that newly divided spirilla have flagella only at one end and grow a new tuft from the other end before they divide again. Spirilla bearing flagella bundles of different lengths, probably representing stages in the growth of a new tail, are illustrated in Fig. 10. Recently Stocker (1957; Stocker and Campbell, 1959) has followed the growth of new flagella in suspensions of *Salmonella* cells whose original flagella had been sheared off by a propeller rotating at high speed. He noted that the bacteria which had been deprived of their flagella were not moving about but started to swim again at the same time at which stained preparations showed that new sets of flagella had begun to be formed. Stocker noted an informative correlation between the length and number of flagella left or grown out again and the type of movement of the bacilli bearing these flagella: "After two minutes treatment only 16% had flagella of length 2 μ, less than 1% showed translational motility and 16% were rotating" (Stocker, 1957). When flagella reappear in undisturbed cultures they are at first short and then longer, and Stocker noted that the cells showed first rotational and then translational motility. He states that "rotation is probably caused by the activity of very short flagella, these are either residual stumps, or new flagella growths" and concludes: "Thus non-lethal treatments which remove flagella cause loss of motility as would be expected if their flagella are indeed the locomotor apparatus of flagellated motile bacteria."

A different view of the nature of flagella has been urged in many publications by Pijper (1957) to whose motion pictures of swimming bacteria we owe much of our knowledge of the behavior of flagella *in vivo*. According to Pijper, bacteria move because their bodies contract. Their flagella are mere passive appendages, the product rather than the cause of motility. It is admittedly difficult to understand at first glance how the action of the tenuous flagella can propel the much larger and heavier bacteria through a medium of the viscosity of water, and at least one eminent British physicist, on seeing some of the first electron micrographs of flagella, declared it to be impossible. There are nevertheless many observations apart from the very suggestive ones of Stocker (1957; Stocker and Campbell, 1959), which are best explained by regarding flagella as the organs of the motility of flagellated bacteria.

It has been shown, for example, that the flagella of bacteria immobilized in a thin film of moisture beneath a coverslip continue to lash about for a long time, sweeping in this way a clear area around them in a film of India ink (Ørskov, 1947; Kingma Boltjes, 1948) or imparting a violent whorling motion to small carmine particles in their vicinity (Mallett *et al.*, 1951). If flagella resembled stiff wires then one would expect them to swing for a few moments after the bacterium carrying them has been stopped in its path, and Pijper has used this analogy to account for the "residual" movements of flagella in experiments in which carmine particles are used to detect them. But this explanation overlooks the length of time, half hours and longer, during which continuous, active, independent movements of the flagella of immobilized bacteria have been observed. A challenge to our understanding of bacterial motility is presented by the "paralyzed" mutants of *Salmonella* species which cannot swim, but are flagellated nevertheless (Hirsch, 1947). Flagellated but immobile bacteria have also been obtained in experiments in which mechanically deflagellated cells of S. *typhimurium* were induced to regenerate their flagella in an amino acids, salts, glucose medium in which fluorophenyl alanine had been substituted for alanine (Kerridge, 1959). The flagella of the paralyzed bacteria were of abnormal shape in having a wavelength only half that of the control. In the absence of the analog and with alanine present, the same medium readily permitted the regeneration of normal flagella and the return to normal motility.

A. *Origin of Flagella*

Flagellin, the protein of flagella is not a part of bacterial cell walls, and flagella remain attached to the protoplast when the cell wall is dissolved by the action of lysozyme (Weibull, 1958). Both kinds of observation suggest that the origin of flagella is in the interior of the cell. However, little is known of the way in which flagella are anchored in the bacterial protoplasm and about their manner of growth and regeneration. Something has been learned from the electron microscopy of bacteria whose cytoplasm had been largely destroyed by autolysis or by digestion with trypsin (Bradfield, 1956; Pease, 1956; Grace, 1954). Flagella are very resistant to either of these treatments and sometimes can be traced back to their origins in the remains of the disintegrated cells. The dense, more or less spherical bodies which have been found at the base of flagella of *Proteus* (Houwink and van Iterson, 1950) and of several polarly flagellated bacteria probably consist of the same autolysis- and digestion-resisting proteins as the flagella themselves. Since nothing is known about their fine structure, they cannot yet be compared with the roots of plant and animal cilia.

The inheritance of functioning flagella by bacteria which are genetically incapable of making new ones (Stocker, 1956) and the unimpaired activity of flagella in bacteria whose cytoplasm has all but disappeared in the course of spore formation (Robinow, 1957) indicates a remarkable autonomy of these organelles. The sources of energy for the activity of the flagella are not precisely known, but in one instance a good case has been made out for adenosine triphosphate (ATP) (Sherris *et al.*, 1957).

Bacteria may also have other appendages which are straight, shorter and thinner than flagella, and chemically different. Not concerned with motility, these so-called "fimbriae" (Duguid *et al.*, 1955; Duguid, 1959) endow their bearers with a stickiness that makes them agglutinate red blood cells. Fimbriae and flagella may occur side by side on the same bacterium.

VII. MOTILE, NONFLAGELLATED BACTERIA

Several groups of bacteria, the sulfur-storing forms belonging to the genus *Beggiatoa, Vitreoscilla* (Pringsheim, 1951), *Thiothrix* (Harold and Stanier, 1955), and the myxobacteria are capable of gliding on surfaces but lack visible organelles that might be regarded as the cause of their movements. The absence of contractile elements is particularly puzzling in the myxobacteria, for these not only glide, but are capable also of rapid changes of shape.

VIII. SPIROCHETES

Most spectacular among motile microorganisms are the large free-living spirochetes whose violent unceasing writhings soon attract the attention of the microscopist of putrid waters (Fig. 13). The motility of parasitic forms ranges from the ponderous progress and frequent angular bending and unbending of the spirochete of syphilis, recently analyzed afresh by Sequeira (1956), to the dizzy spinning of slender *Leptospira* on its long axis. With the exception of the large *Cristispira* (Dobell, 1912), most spirochetes have proved too fine for rewarding light microscopy, but interesting observations have been made with the electron microscope (Bradfield and Cater, 1952). What follows is chiefly based on the

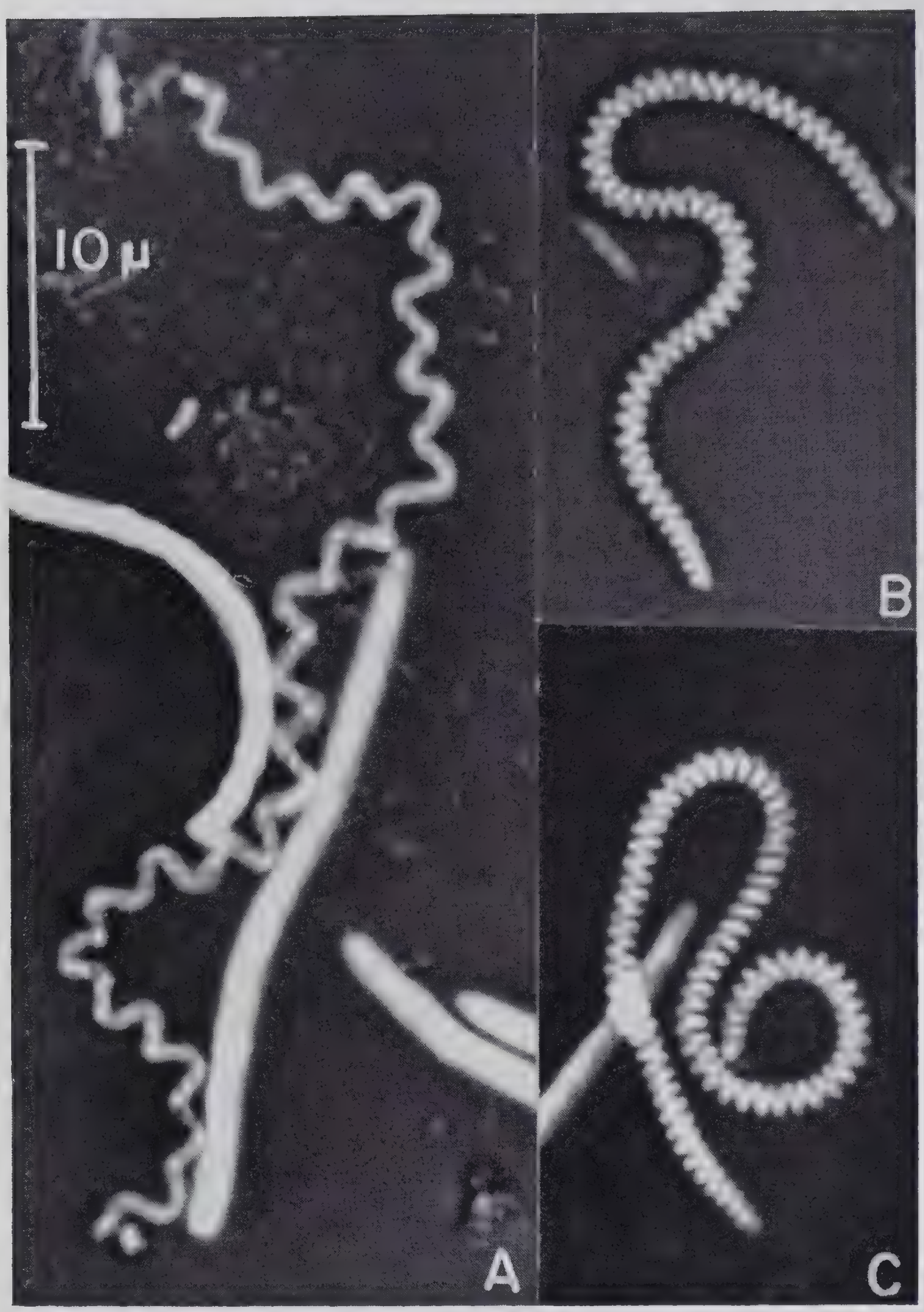

FIG. 13. Free-living spirochetes. A. Probably *Spirochaeta plicatilis* accompanied by a large straight bacterium and a spirillum. B and C. Two individuals of an extremely finely coiled, hitherto undescribed, species. Relief staining with nigrosin.

work of Swain (1955, 1957). The cytoplasm of *Leptospira* is wound around a straight rod (Fig. 14, B). Both are enclosed by the cell wall. The axial rod serves as an internal flexible stiffener of the helically shaped cell. It is not known whether it has a fibrous fine structure and whether it is contractile. In other spirochetes the place of the axial rod is taken by fibers—three in *Treponema,* eight to twelve in *Borrelia,* in both approximately 200 A. thick—which are arranged in the shape of a closely packed, possibly contractile cable or ribbon inside or outside (?) the cytoplasm (Fig. 14, A). The characteristic waves of *Treponema* and *Borrelia* are lost or flattened when the fibers of the axial cable are broken and displaced. The work of Swain has made it seem probable that earlier reports of externally borne flagella in spirochetes were based on imperfectly preserved specimens in which the axial cable had suffered some dismemberment. External flagella are not found on carefully preserved specimens. The fairly quick destruction of killed spirochetes by pepsin and trypsin suggests that their cell walls are chemically different from those of bacteria.

IX. PLASMA MEMBRANE AND CYTOPLASM

An excellent discussion of plasmolysis and accounts of quantitative experiments will be found in a masterly survey by Mitchell and Moyle (1956) and in Mitchell (1959). Precise details of the permeability of protoplasts and the composition of the plasma membrane will be found in the same articles and in many important contributions to this subject made by Weibull (1955a, b; 1958; Weibull and Bergström, 1958; Weibull *et al.*, 1959). Evidence for a cytoplasmic membrane regulating the flow of materials into and out of bacterial cells has been obtained from experiments on plasmolysis and from studies of living bacterial cells released from their walls.

A. *Plasmolysis*

Bacteria can be plasmolyzed. The protoplasm of gram-negative bacteria such as spirilla or *Escherichia coli* pulls away from the cell wall when they are taken from water into 0.25 M solutions of sucrose NaCl or KNO_3. Detailed protocols of many such experiments and clear illustrations of the effects observed have been given by Albert Fischer (1895). The staphylococcus shrinks by 6–7% by volume when it is taken from 0.1 M to 1.0 M NaCl, and the same coccus as well as the equally gram-positive *Sarcina* and *Bacillus megaterium* have been seen to contract when they are transferred from 0.1 M NaCl to 0.1 M NaCl containing 1.2 M sucrose (Mitchell and Moyle, 1956). This shrinkage is evidently the

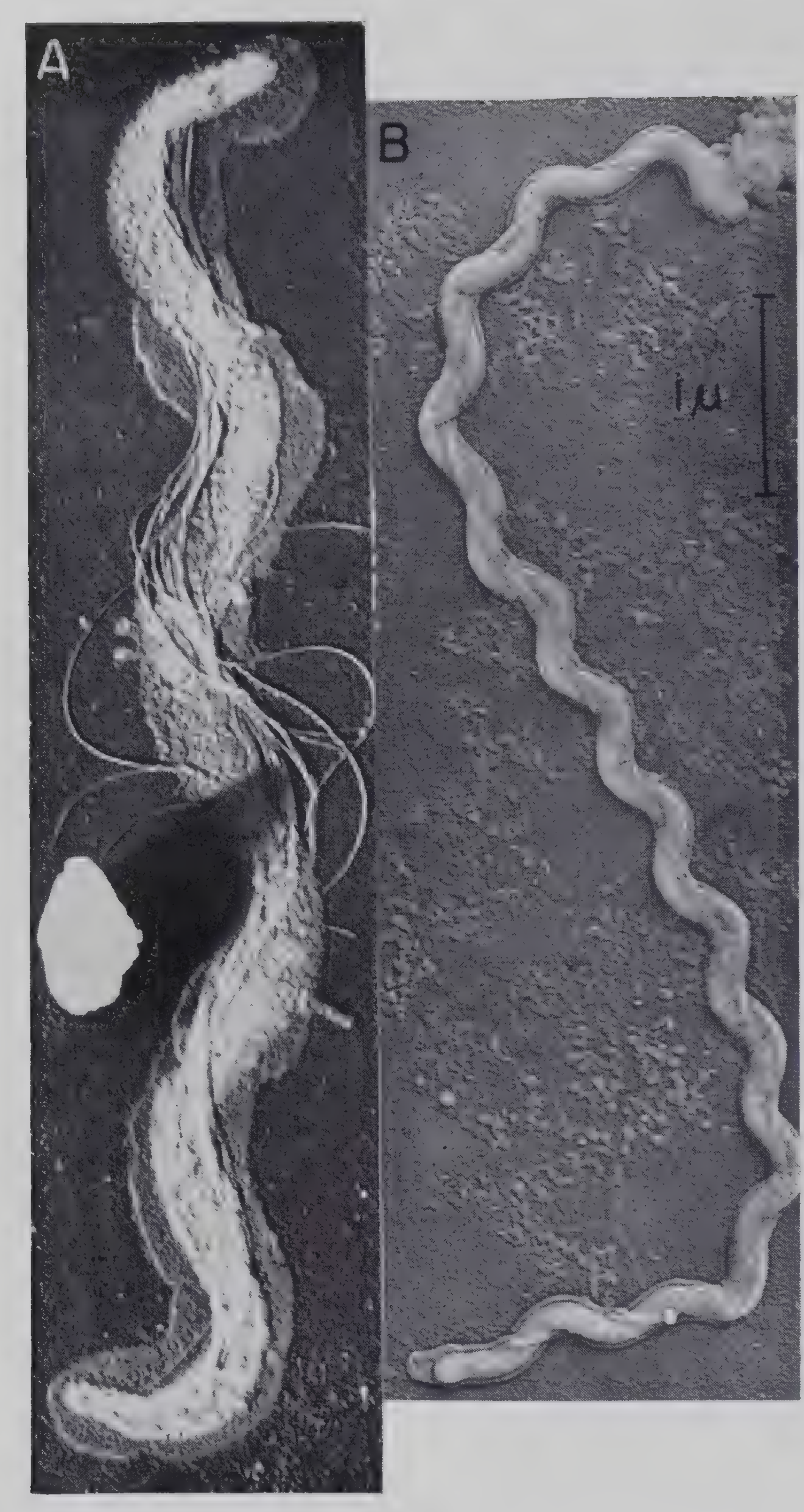

FIG. 14. Electron micrographs of spirochetes. A. A small spirochete from the cecum of the guinea pig. An intracytoplasmic cable of fibrils has partly unwound itself. It is still intact in the lower half of the picture. B. *Leptospira canicola:* the cytoplasm is wound around an axial rod; both are surrounded by the cell wall. A. Courtesy of Dr. W. van Iterson; B, reprinted, with permission, from Swain (1957).

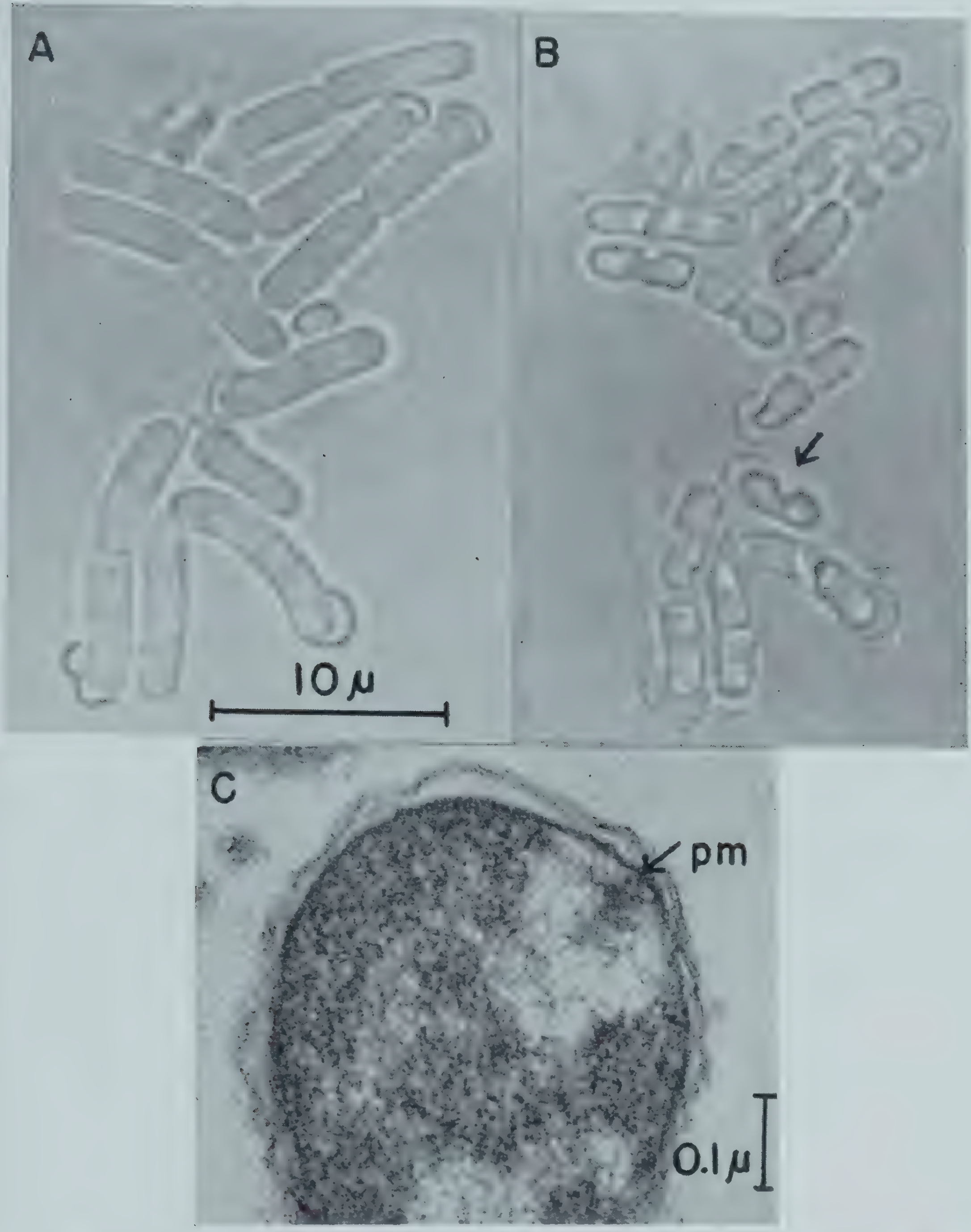

FIG. 15. Plasmolysis in *Bacillus megaterium*. A. Living cells recently grown from spores and with spore coats still attached. B. The same group a few minutes after exposure to 25% (about 4.4 *M*) sodium chloride in water. The bacilli have become shorter and narrower and in many places the protoplasts have pulled away from the cell wall—markedly so in the short bacillus indicated by the arrow. C. Electron micrograph of a section of *Escherichia coli*. The cell wall is thrown into folds. Beneath it can be seen the plasma membrane (*PM*). Reprinted, with permission, from Kellenberger *et al.* (1958).

equivalent of the plasmolysis of gram-negative bacteria. It is apparently not due to dehydration because it does not happen when sucrose is replaced by glycerol of comparable molarity. Separation of cytoplasm and cell wall, so readily provoked in gram-negative species, has never been observed in spherical gram-positive forms, and very high concentrations of sodium chloride, 2–4 M, are required to produce it in rod-shaped gram-positive bacteria. At these concentrations there is both considerable shrinkage and a partial detachment of the cytoplasm from the cell wall (Fig. 15, A, B). Plasmolysis of *Bacillus* cells is reversible and may be repeated many times (Imsenekij, 1937), but it has not yet been recorded whether bacilli are capable of recovery and renewed growth after several courses of plasmolysis. The need for high concentrations of salts in these experiments probably reflects, as Mitchell and Moyle (1956) have pointed out, not so much excessively high internal osmotic pressures, as very firm adhesion of the cytoplasm to the cell wall. It is this firm bonding which probably accounts for the fact, true also of plasmolyzed gram-negative bacteria and apparent in Fig. 15, B, that the cytoplasm rarely pulls away from the wall with the whole of its surface but usually does so only here and there in the form of short lengths of "concave clear zones at the border of the shrunken protoplast" (Imsenekij, 1937). Glycerol and other small, nonionized organic molecules, in concentrations comparable to those at which salts cause plasmolysis, readily enter bacterial cells and therefore fail to plasmolyze them. Taken together, these and numerous other observations suggest that there is a selectively permeable barrier or plasma membrane at the surface of the bacterial cytoplasm. In intact gram-positive bacteria the site of what may well be the cytoplasmic membrane is revealed by Victoria blue B or 4B, basic phenyl methyl dyes which on first contact with living or fixed bacteria leave the cell wall unstained and penetrate only slowly into the cytoplasm but endow its surface with sharply drawn blue contours (Robinow and Murray, 1953). Visible traces of a cytoplasmic membrane have been seen in electron micrographs of *Spirillum serpens* (Chapman and Kroll, 1957), *B. cereus* (Murray, 1957), *B. subtilis* (Tokuyasu and Yamada, 1959a), and, very clearly, in phage-infected cells of *E. coli* (Kellenberger and Ryter, 1958) and in an unidentified bacterium recently described by Chapman (1959). The best evidence of the existence of an osmotic barrier at the surface of the cytoplasm has been obtained in studies of the permeability of stabilized bacterial protoplasts released from their walls (Weibull, 1955a, b; Weibull and Bergström, 1958; Weibull *et al.*, 1959). These fragile products of the laboratory behave like osmometers, are about as impermeable to sucrose and phosphate as intact bacilli, and when left to disintegrate in hypotonic solutions leave behind more or

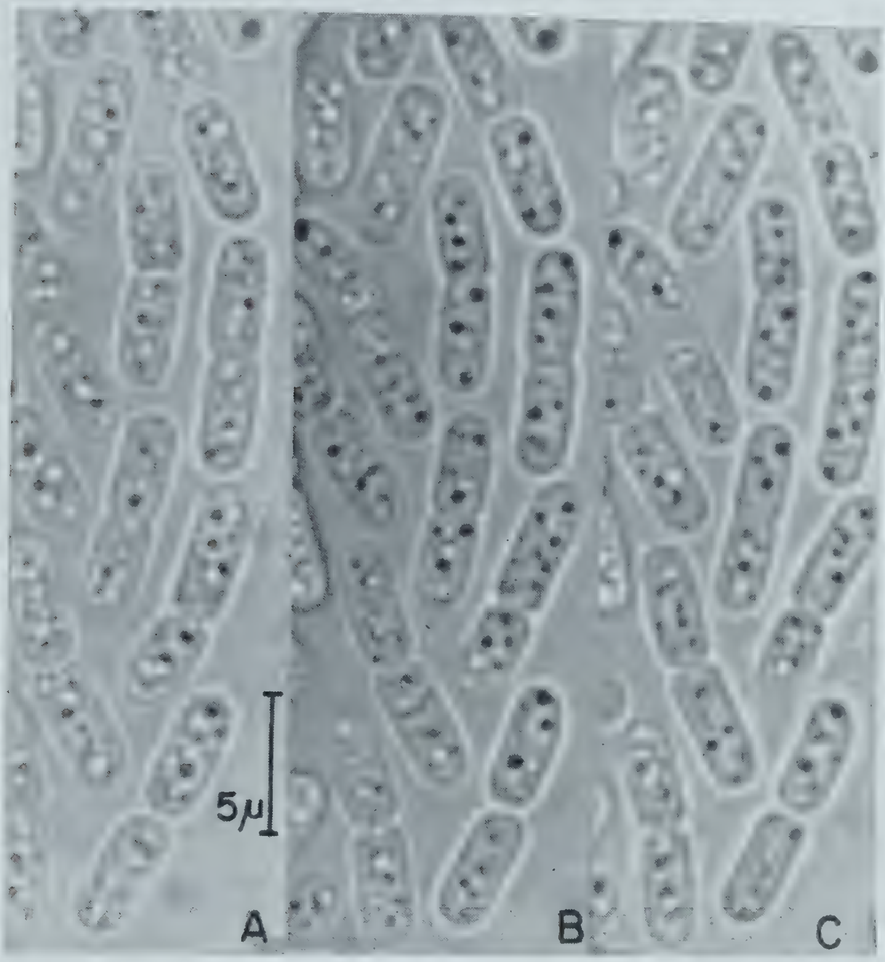

FIG. 16. Three photographs of the same living cells of *Bacillus megaterium* taken at intervals of 4 minutes to show changes in the relative positions of lipid droplets. This is the same slide culture that, 18 hours earlier, provided the sequence shown in Fig. 6.

less empty, thin-walled "ghosts" whose composition (mainly lipoprotein) differs markedly from that of bacterial cell walls but fits that of the osmotic barrier whose existence had already been inferred from permeability studies on intact bacteria and intact protoplasts.

B. The Cytoplasm. Mitochondrial Function of the Plasma Membrane

The physical state of the ground cytoplasm of bacteria is difficult to assess because at the time of most rapid growth it is usually evenly transparent and lacks visible internal organization. Phase contrast microscopy reveals that the nuclei of *B. cereus* var. *mycoides* undergo changes of shape which are not obviously connected with their process of division. These movements are perhaps passive and caused by currents in the cytoplasm. Continuous changes in the relative position of droplets of

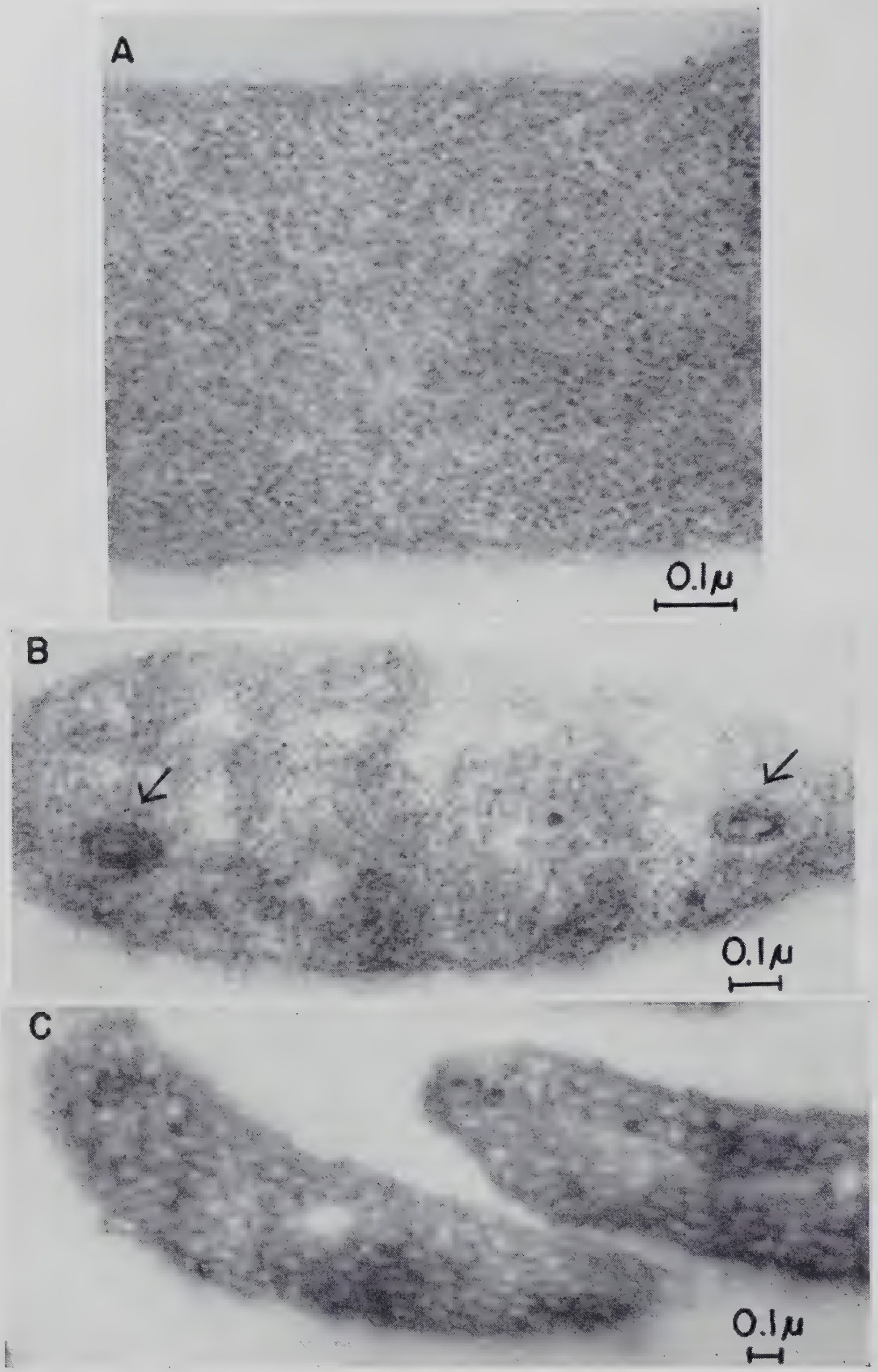

Fig. 17. Electron micrographs of bacterial cytoplasm. A. A highly resolved micrograph of the cytoplasm of *Spirillum serpens*. Apart from an area of low density in the center of the picture which may represent chromatin, the cell is evenly filled with closely packed grains and short lengths of dense material with diameters around 150–200 A. B. *Spirillum serpens:* Examples of small complexes of concentric membranes ('twirls') which are now being found in many bacteria. C. Sections of *Rhodospirillum*. The cells are crammed with profiles of chromatophores. A–C from electron micrographs prepared by Dr. R. G. E. Murray.

lipid have several times been described in cells of *B. megaterium* (von Klinckowstroem, 1931; Bayne-Jones and Petrilli, 1933). Such random excursions, which are much slower than those seen in Brownian movement, become very obvious after a few minutes of sustained observation of one and the same lipid-laden cell (Fig. 16). These movements suggest that the cytoplasm of *B. megaterium*, in that phase of its growth when it is crammed with lipid, is in continuous churning motion. Growing bacteria are rich in ribonucleic acid (RNA) (Vendrely and Lipardy, 1946), strongly absorb ultraviolet light (Malmgren and Hedén, 1947), and lose their normally high basophilia when they are digested with ribonuclease (Tulasne and Vendrely, 1948). Their RNA content varies considerably with the rate at which bacteria multiply and is low at the end of the phase of growth (Malmgren and Hedén, 1947; Caldwell *et al.*, 1950; Caldwell and Hinshelwood, 1950). In electron micrographs of sections, the cytoplasm of many bacteria looks uniformly granular, but systematic comparisons of the state of the cytoplasm at different phases of growth of the same bacterium have, I believe, not been carried out. The cytoplasm of tubercle bacilli has several times been found to contain many large vesicles and dense granules of varying sizes (Knaysi *et al.*, 1950), but the most differentiated bacterial cells to be described so far are those of photosynthetic bacteria (Vatter and Wolfe, 1958; Hickman and Frenkel, 1959) which, in sections appear filled with circular profiles that correspond in size to the chromatophores that have been isolated by differential centrifugation of mechanically disrupted rhodosprilla (Fig. 17, C). A system of concentric lamellae, resembling in their dimensions those found in blue-green algae (von Wettstein, 1957), fills, surprisingly, the periphery of the cytoplasm of the budding and branching photosynthetic bacterium *Rhodomicrobium* Vatter *et al.*, 1959). In most other bacteria the cytoplasm appears as a mass of densely packed granules about 200 A. in diameter, believed to contain RNA, but is singularly lacking in organelles of familiar constructino (Fig. 17, A). In *B. megaterium* and in the staphylococcus all or most of the recoverable cytochrome is in the plasma membrane and several oxidative enzymes have been found there as well (Mitchell, 1959; Weibull, 1953). At present it seems probable that functions which in the cells of higher organisms are performed by mitochondria are in bacteria dealt with by the plasma membrane (Stanier, 1954). It is probably significant that in the very large bacterium *Thiovulum majus* the plasma membrane sends folds deep into the cytoplasm (Fauré-Fremiet and Rouiller, 1958). It is only a small step from this observation to the current speculation of histologists that mitochondria proper can be formed from invaginations of the plasma membrane of animal tissue cells (Robertson, 1959).

Electron microscopy is now beginning to discover systems of concentric membranes (Fig. 17, B)—some very small, others quite large—in the cytoplasm of bacteria, especially tubercle bacilli (Glauert quoted in Murray, 1959, see Plate II, Figs. 11 and 12; Zapf, 1957; Shinohara *et al.*, 1958). Striking formations of this kind have been discovered in *Streptomyces coelicolor* (Glauert and Hopwood, 1959). There are indications that all these organelles are not the same. Perhaps the smaller and most superficial ones of these structures are only temporary infoldings of the plasma membrane. The larger sytems (Figs. 28, 34) have the look of proper organelles and by some are regarded as the bacterial equivalents of mitochondria, a plausible inference from the observation that some of them are continuous with the plasma membrane, which is known to be rich in oxidizing enzymes and cytochrome (Glauert and Hopwood, 1959).

Quantitative observations are now required to link these findings to the earlier work of Mudd and his collaborators (1953, 1954), who have found with the light microscope discrete foci of oxidative and other chemical activity in many different bacteria (though often not at the time of the most rapid growth) and have regarded these as reflecting the activity of mitochondria-like organelles.

A beginning has been made in the work of Niklowitz (1958), who has found that in *E. coli* tetrazolium is reduced at sites closely adjoining certain, not yet clearly resolved, relatively large, dense bodies which he has often found near one end of the cells in micrographs of sections. So far he has found them only in cultures past the phase of rapid multiplication, which is not what one would expect of an organelle believed to be active in the cell's metabolism. Similar structures have been encountered and tentatively identified as mitochondrial equivalents in *E. coli* K12 S after it had been suspended for 1 hour in buffered saline and also in cells infected with phage (Ryter and Kellenberger, 1958).

X. The Nuclei of Bacteria (Figs. 18–25)

A. *Light Microscopy. The Process of Division*

The literature on bacterial nuclei is large and controversial. It has been reviewed several times in recent years (Piekarski, 1949; De Lamater, 1953a; Delaporte, 1956; Robinow, 1956a). Most aspects of this subject are thoughtfully discussed on the basis of long first-hand experience by R. G. E. Murray (1960) in a new treatise, "The Bacteria." No fresh summing up is required at this point.

Direct observation soon leads us to the main problems. Many bacteria possess relatively large organelles which are less dense than the cytoplasm, grow and divide, give a positive Feulgen reaction (Figs. 18, 19), show

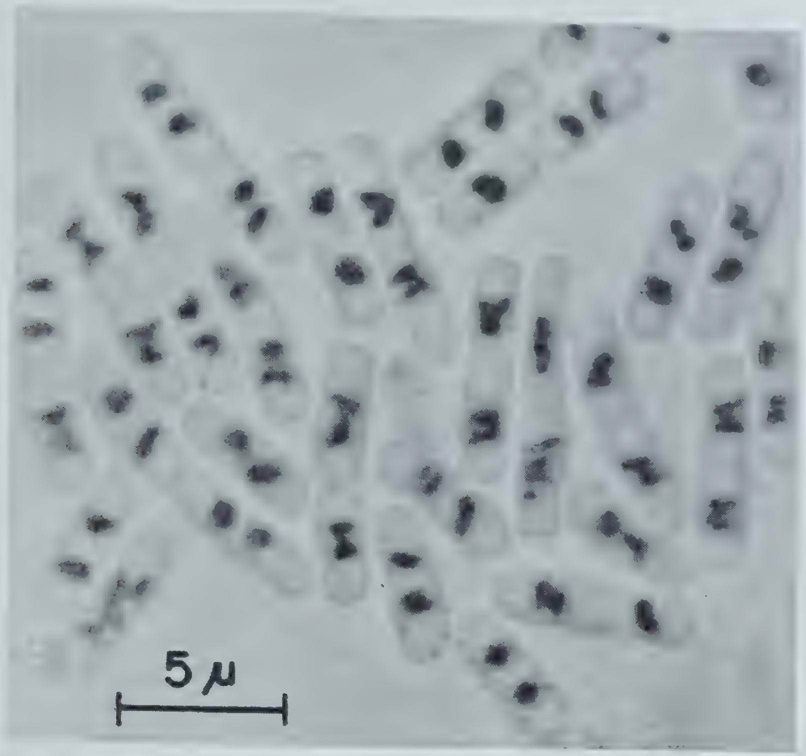

FIG. 18. Positive Feulgen reaction of the chromatin of *Bacillus cereus*. Osmium tetroxide vapor fixation. No counterstain. Photographed mounted in water. The difference between nuclei and cytoplasm has been accentuated by inducing the nuclei to contract into more compact shapes than they usually have. This was done by transferring the bacilli 10 minutes before fixation from a medium with 0.5% NaCl to one with 3.5% NaCl. The contraction of the chromatin is a transient affect of the change in salt concentration (Whitfield and Murray, 1956). It does not impair the viability of the bacteria and is comparable, in principle, to the result of techniques now in use to enhance the visibility and facilitate the counting of animal chromosomes.

green fluorescence when stained with acridine orange (Anderson, 1957), and, sometimes directly (Piéchaud, 1954; Hartman and Payne, 1954), but more reliably after special treatment, stain like chromatin. They do so most readily with Romanovsky dyes, indispensable to the parasitologist because of their affinity for the otherwise hard to stain chromatin of malaria parasites. They have been called various names, e.g., *éléments nucléaires,* nucleoids, chromosomes, chromatin bodies, and, with suitable connotations of vagueness and plasticity, "nucleoplasms" (Ryter and Kellenberger, 1958; Kellenberger *et al.*, 1958). Despite my own preference for "chromatin bodies" (Robinow, 1956a) or just "chromatin" I shall, for the sake of simplicity, frequently use the term "nucleus" in these pages. I wish to make it plain at the beginning that there are many different types of nuclei, that in my opinion bacterial ones are quite unlike those of plant and animal cells and unlike those of most other protists, and that "nucleus" in the present context will only be employed as a con-

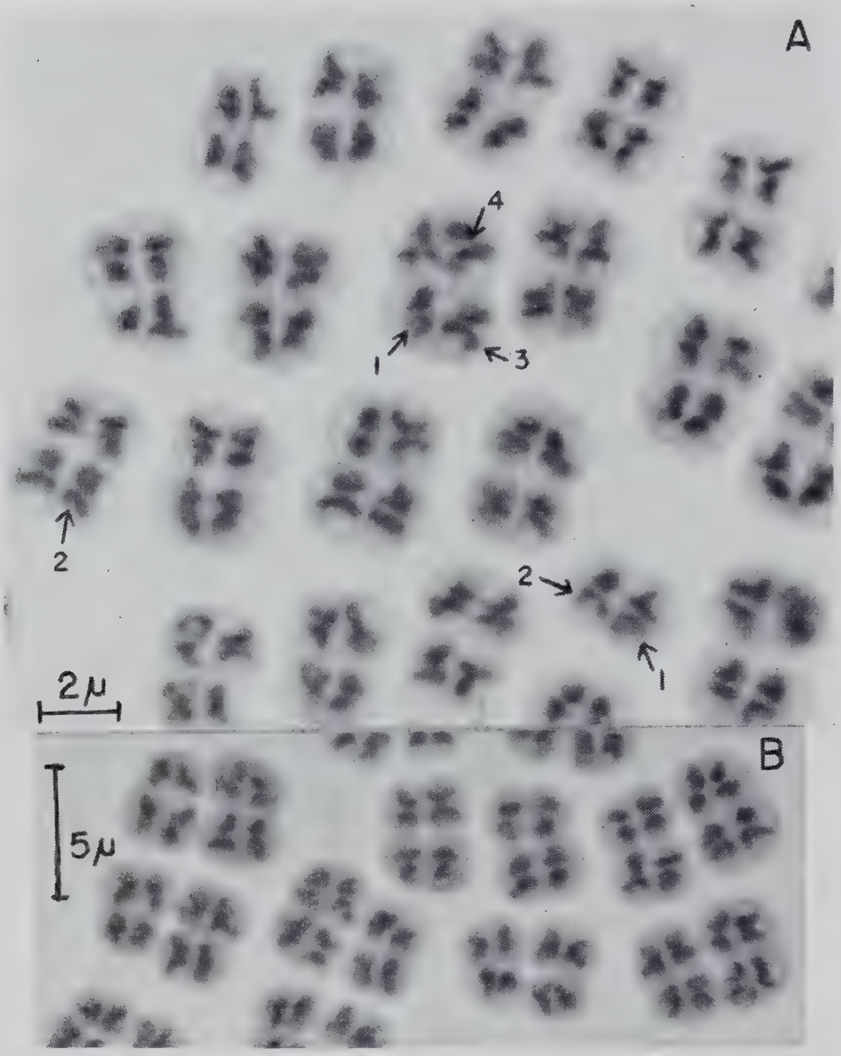

FIG. 19. Well-defined compact nuclei in cells of the same tetrad coccus that provided Fig. 1. Osmium tetroxide vapor fixation. A. Azure A–SO_2 stain. B. Feulgen, mounted in acetocarmine; 1–4, successive stages in the division of the nuclei. The correctness of this sequence of events has been confirmed by phase contrast microscopy of living cocci.

venient piece of laboratory jargon meaning something that probably performs some of the functions of a nucleus but about whose organization and behavior during division we are still largely in the dark.

Two questions of general biological interest might be asked about bacterial nuclei: What is their organization and how do they multiply? The fine structure of bacterial chromatin may be examined about as easily in one species as in another, but not all bacteria have nuclei likely to provide us with useful answers to the second part of our question. In some bacteria, e.g., in *Sarcina ventriculi* (Delaporte, 1957; Knöll and Niklowitz, 1958), certain large forms from the intestines of tadpoles (Delaporte, 1959), gliding bacteria of the genus *Vitreoscilla* (Costerton, 1957), in *Shigella* (Whitfield and Murray, 1954), *Azotobacter* (Truant, 1952), perhaps in *E. coli* growing in a liquid medium low in NaCl (Ryter and Kellenberger, 1958) and in protoplasts of *B. megaterium* growing between agar and a coverslip (Fitz-James, 1958), the chromatin appears to be a continuum, a net or spongework, extending evenly through the cell. In such forms the chromatin probably adds to its substance continuously and at many different points and is from time to time roughly divided into two (or more?) parcels, either independently or in step with cell division. Such nuclei are not unit structures and not a good choice for studies of the ways of duplication and segregation of nuclear elements. Large size is not necessarily an advantage here. In the cells of the larger Bacillus species several separate nuclei of tortuous shape are packed closely together. Impressions of the changing phases of these nuclei can be obtained by watching their behavior in the living cell (Fig. 20), but in stained slides and in electron micrographs their complicated shapes and close spacing make the interpretation of their activities difficult. Obviously, these forms too are not the easiest ones for studies of modes of multiplication. Most rewarding from our point of view are nuclei of simple shape that are not too close to each other and multiply in step with the growth and division of the cells containing them. Nuclei of this kind are found in the smaller *Bacillus* species and elsewhere. Those of *B. subtilis* and *B. cereus* will serve as an example.

Phase contrast microscopy of *B. subtilis, B. cereus* var. *mycoides*, and *B. cereus* growing in the presence of high concentrations of gelatin, a technique derived from the work of Barer *et al.* (1953) and first successfully employed by Mason and Powelson (1956), reveals more clearly than do comparable earlier studies (Stempen, 1950) that living bacterial nuclei lack sharp boundaries and have a soft, even, cloudlike structure. If they have parts, these must be very small compared with the nucleus as a whole. The process of division is simple and direct. Two phases can be distinguished: a compact one in which the nuclei are well defined and shine brightly in dark phase contrast and in acridine-stained films fluorescing in ultraviolet light (Anderson, 1957) and a more diffuse one in which the nuclei are less luminous by both techniques. Time lapse phase contrast micrography shows that the bright nuclei are those that are preparing to divide and that they become diffuse, broader, and, in some instances, more tortuous during the segregation of the duplicated materials into two new nuclei (Figs. 20, 21). The study of nuclear division in stained preparations of germinating spores of *B. megaterium* had already suggested some time ago that "recently divided nuclei" (the same as "nuclei preparing to divide" in the present context) are compact and

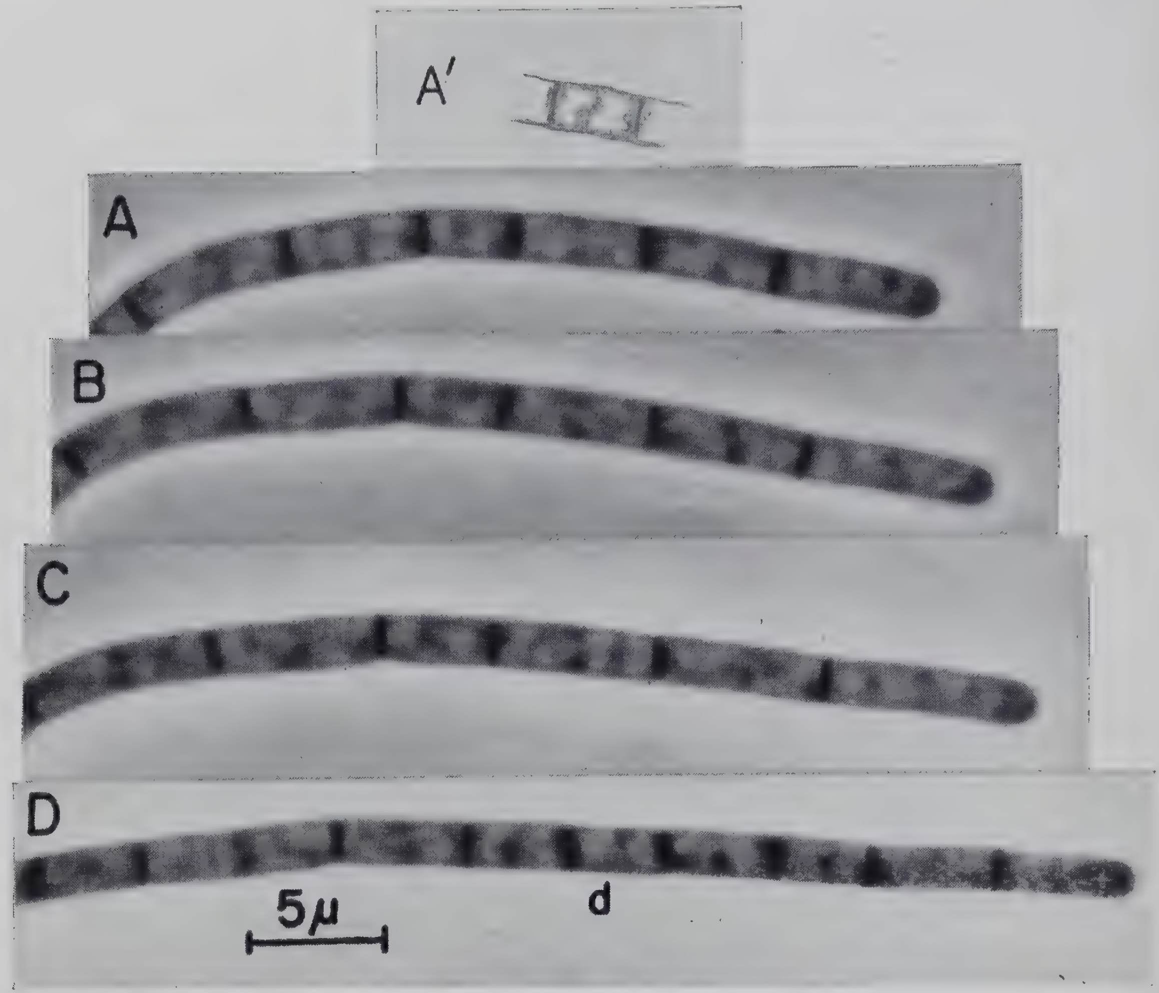

FIG. 20. Time lapse photographs, 10 minutes apart, of a chain of cells of a large bacillus (strain Medusa of this laboratory) growing in heart infusion agar with 16% gelatin. Phase contrast microscopy; magnification: ×2800. The four panels have been arranged so as to keep the third cell from the right in the center of the figure and with no regard to the relative movements of points of reference. The increment in length of the chain from A to D is in reality greater than it appears to be in this illustration. In A and B the third cell from the right contains two nuclei of complicated shape. In B they are large, of open design, and rather indistinct. Ten minutes later, in C, the right nucleus has divided into two compact shapes which stand out clearly against the cytoplasm. The left nucleus has also divided, but its daughters are not yet as luminous as those of the nucleus at the right. They have become so in D, and now each pair of daughter nuclei lies in a cell of its own. Brightness means incipient duplication, and in one of the daughters of the right nucleus that process has already begun. Experiences with other living bacilli justify extension of the sequence backward in time and the assumption that some 30 minutes before panel A was taken the two nuclei in the third cell from the right, which we have been following, were in the same state in which we are leaving their four daughters in D. A sketch (A′) illustrating this assumption has been added to the photographs. The reader is advised to return to this figure after he has studied the illustrations of dividing nuclei in *Bacillus subtilis* and *B. cereus* which follow.

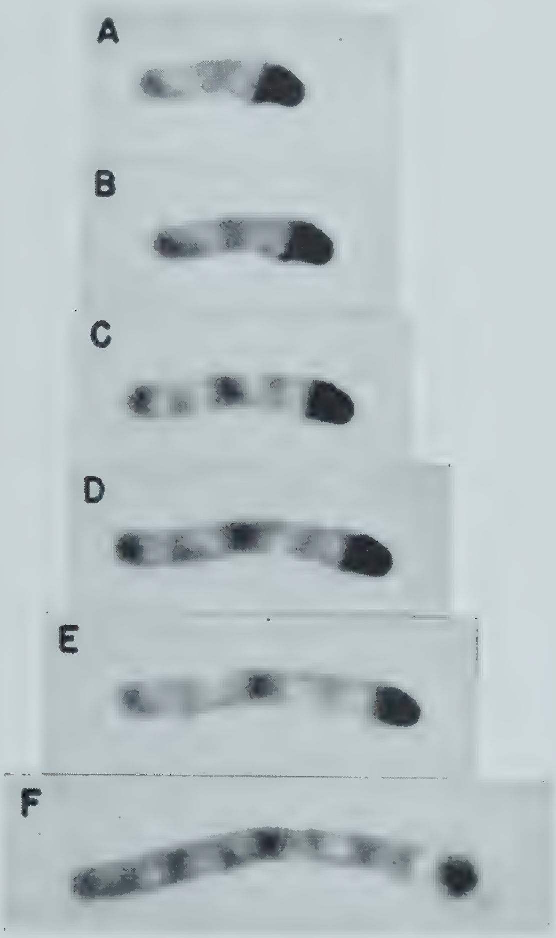

FIG. 21. Time lapse, phase contrast micrographs of steps in two successive divisions of the nuclei of a *Bacillus subtilis* growing in heart infusion broth with 20 % gelatin. The interval of time between A and F is 34 minutes. Note how the same four nuclei that have relatively sharp contours and contrast strongly with the cytoplasm in C, have become broader, hazy, and less luminous in E. Stage C corresponds to the preparation of the division of the nuclei. Division is well under way in E and completed in F. The new nuclei are once again compact and more clearly set off from the cytoplasm than those of the immediately preceding stage. The recurrent changes in size, sharpness of contours, and luminosity of the nuclei imply underlying changes in fine structure still waiting to be recognized in electron micrographs.

that nuclei of more open and complex appearance are nuclei in a state of division (Robinow, 1956b). For *B. subtilis* and several other bacilli this has now been confirmed by the microscopy of living cells. These observations dispose of a long-felt and at times overemphasized difficulty. That is the impression, gained from stained specimens, that the texture of the chromatin never changed from one division to the next (Robinow, 1956a, b). The recurrent changes of the optical properties of the *living* nuclei that have been observed prove that the chromatin *does* undergo a change in fine structure before division and raise the hope that the

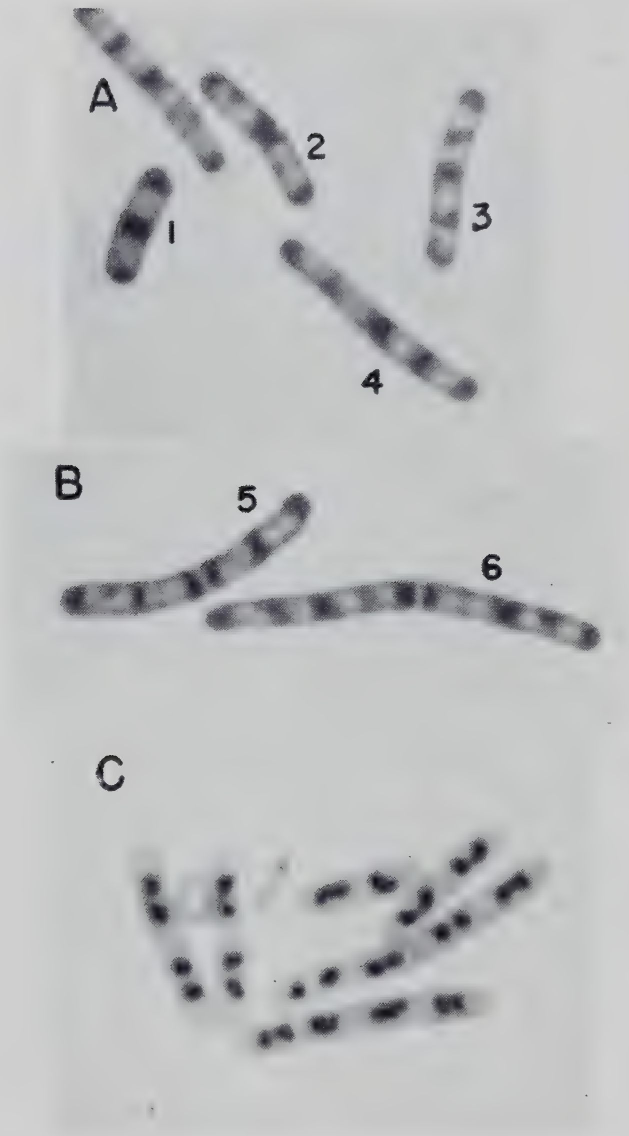

FIG. 22. A. and B. Nuclear division in *Bacillus subtilis* traced in fixed and stained preparations. A, B, 1–6 reflect a sequence of nuclear changes visible in this bacillus during life and illustrated in Fig. 21. In A3 the nuclei are compact and stand out sharply from the cytoplasm. In A4 they have become larger and now their contours are vague. Division is well under way in B5 and completed in B6 where most of the nuclei are again as compact as those in A3. Magnification: ×3600. Fixed *in situ* with Bouin. Stained 40 seconds with 0.1% thionine. Photographed mounted in water.

C. Nuclei of *B. subtilis* stained by the OsO_4, HCl, Giemsa procedure. Comparison with Fig. 21 shows that the nuclei have shrunk. Differences in texture between different stages of division, comparable to those seen in Figs. 20 and 21 are not apparent.

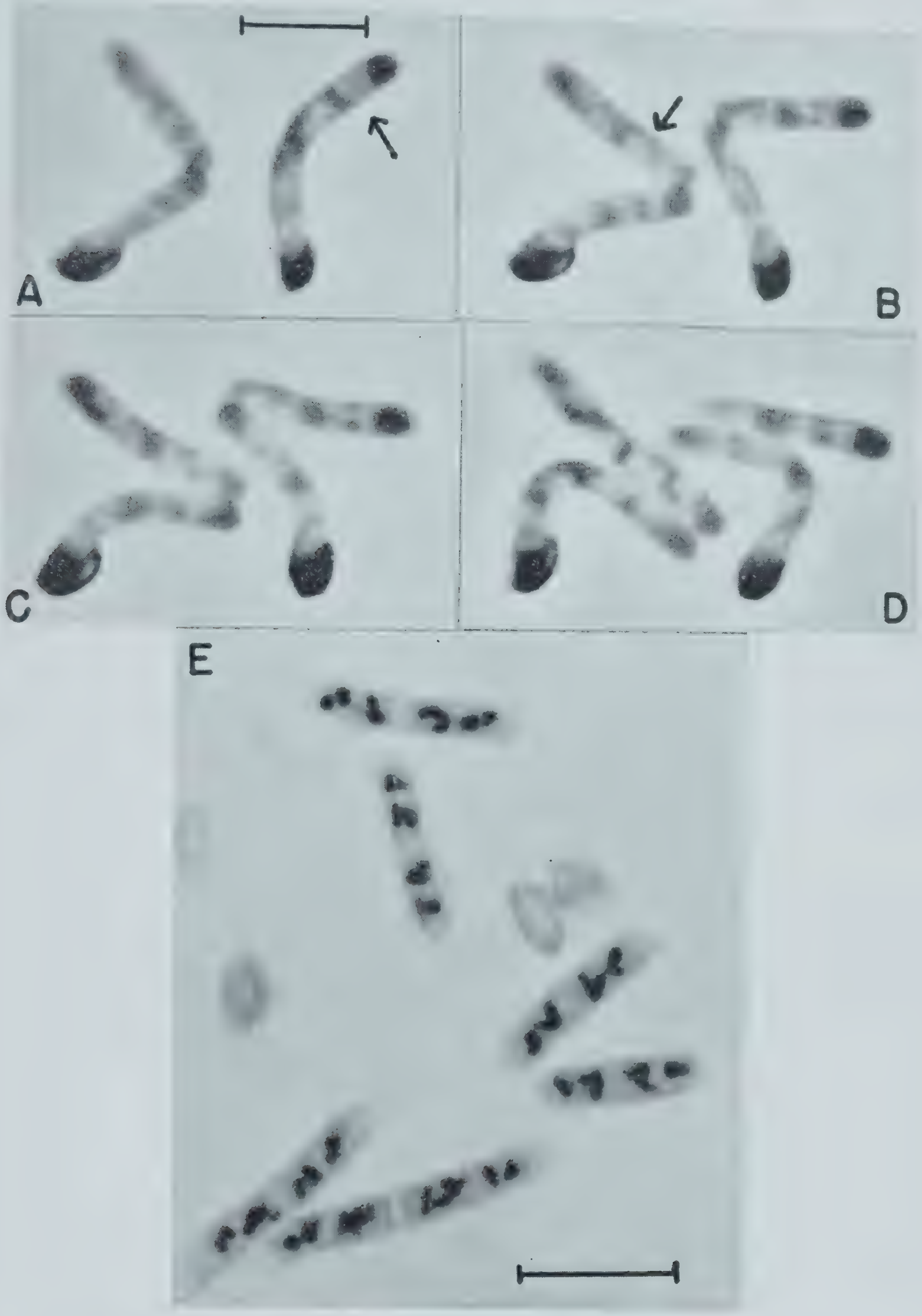

FIG. 23. *Bacillus cereus* var. *mycoides.* A–D. Time lapse, phase contrast micrographs taken at intervals of 9 minutes. The arrow in A points to a pair of bright nuclei, products of a very recent division. They move apart in pictures B and C. In D both have become broader and softer of outline. They are now in the process of renewed division. The arrow in B points to a vague triangular nucleus which has become brighter and larger in C, and in D has divided into two bright compact nuclei which will now follow the course of the two indicated in A. Note the odd shapes of the nuclei and their variable orientation relative to the long axis of the bacilli. This is typical of the pattern of chromatin in *B. cereus* var. *mycoides.* E. Osmium-fixed, hydrolyzed, and Giemsa-stained cells of the same strain of *B. cereus* var. *mycoides.* There is good correspondence between the ranges of sizes and shapes of the nuclei of the living bacilli in A–D and the killed ones in E. The scales represent 5 μ.

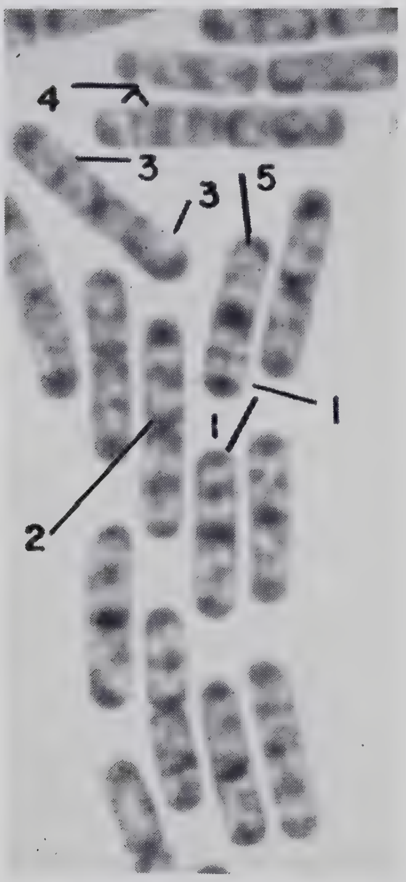

Fig. 24. Steps in the division of the nuclei of *Bacillus cereus* (which closely resembles *B. cereus* var. *mycoides*) traced in a fixed and stained specimen. Bouin, 0.01 % thionine for 40 seconds. The nuclei are not stained and appear white or gray. Single, narrow nuclei (*1*) with sharp contours broaden and soften (2), become more complex (3) and divide into two new narrow nuclei (*4*). At (5) one of such a newly created pair has already begun to divide again. Compare these nuclei and the cycle of their division with the sequence illustrated in Figs. 20 and 21.

division of at least some bacterial nuclei will soon become intelligible in terms of the behavior of their constituent elements. In *B. subtilis* and *B. cereus,* the nuclei of osmium-fixed, hydrolyzed preparations are appreciably smaller than they are during life (Fig. 22). In the experience of this laboratory this effect is due to the action of osmium tetroxide rather than to acid hydrolysis. Correspondence between living and fixed and stained nuclei is reasonably good in *B. cereus* var. *mycoides* (Fig. 23). Observations on living nuclei confirm what the writer has pointed out before, namely, that the volume of the nuclei, their vagaries of shape as well as the subtle differences between nuclei in the various stages of division, are well preserved by fixation *in situ* with the reagent of Bouin (Robinow, 1956b) (Figs. 22–24).

B. Electron Microscopy

Over the last half-dozen years many sections of bacteria have been studied in electron microscopes and different observers have found in them very different things. All are agreed that the nuclei lack a distinct envelope, but in some specimens the nuclei appear finely fibrous (Chapman and Hillier, 1953); in others they are filled with a sparse network of coarse fibers of varying thickness (Birch-Andersen *et al.*, 1953; Tomlin and May, 1955). Others again show the nuclear vacuole only partly filled with a few angular slabs and ribbons (interpreted as chromosomes), as dense or denser than the cytoplasm (Piekarski and Giesbrecht, 1956). Combinations of any of these features and irregular lumps together with fibers and granules, both large and small, have also been obtained. These results are unsatisfactory on several counts. Very inhomogeneous nuclei, so often illustrated, are unconvincing because we know the nuclei to have a smooth and even texture in life. Moreover, Dr. R. G. E. Murray (1958) has shown that the coarser ones among the various published patterns can be matched by frank artifacts than can be produced by suddenly raising the concentration of sodium chloride around the bacteria and fixing them shortly afterward. In other illustrations the nuclei are fine-grained enough but still leave one's curiosity unsatisfied because nothing in them stands out with the clarity and constancy which one expects of basic nuclear elements, and none of them give a clue to what happens in a nucleus when it divides.

Some of the shortcomings of current techniques have recently been made good by improvements worked out by E. Kellenberger and his associates (1958; Ryter and Kellenberger, 1958). Noting that one fixative, osmium tetroxide, has been universally used, they reasoned that the cause of the bewildering variety of published images of bacterial nuclei must lie not in the reagent itself, but in the condition under which it is applied, and they set themselves the task of finding the right ones. Experience gained in a long series of systematically varied experiments has led Kellenberger to recommend fixation in Michaelis buffer at ph 6.2 in the presence of $CaCl_2$ and 0.1% of tryptone or some other mixture of amino acids, to be followed by a wash in buffered solutions of uranyl or lanthanum acetate. Kellenberger, not unlike Whitfield and Murray (1956), sees the bacterial nucleus as a loose gel of DNA, either pure or, perhaps, with its negative charges neutralized by polyamines—at any rate as a form of DNA not combined with protein or histone. These ideas are not shared by all (De Lamater and Echlin, 1959) but the recent demonstration that the bacterium *E. coli* lacks histone (Wilkins and Zubay, 1959) and earlier findings by Belozersky (1947) are in keeping with Kellenberger's hypothesis. Pure DNA does not react with osmium, and, it is argued, since bacterial nuclei lack at the same time the support of a protein skeleton they are liable to collapse and suffer other distortions in the course of fixation with plain osmium tetroxide solutions and the treatments that follow it. The typtone probably forms a strengthening sheath around the DNA fibrils; it is known to react strongly with osmium. Uranyl acetate generally enhances contrast, consolidates the tryptone osmium scaffolding, and somehow prevents its collapse during dehydration and impregnation with methacrylate or polyester resin.

Be that as it may, bacterial nuclei preserved by the "standard fixation" are, in the main, of the right order of size, lack a limiting membrane, usually have irregular contours, and are evenly filled with more or less densely packed fine fibers estimated to have diameters between 30 and 60 A. The maze of fine fibers seen in nuclei after standard fixation resembles that seen in the nuclei of a species of *Pseudomonas* which Hickman (1955) in the course of a systematic exploration of fixation procedures succeeded in preserving in a uniformly and finely fibrous condition.

Standard fixation, then, has several persuasive features: it preserves the nuclei in a state which agrees reasonably well with the range of sizes and shapes and the uniformly low density which are theirs during life and it presents a clearly defined entity, a fibril, as the basic element of the nucleus. The fibril may represent the DNA or some material closely associated with it, or both.

Eventually that fixative will be accepted as most reliable that not only preserves the size and shape of the nuclei, but tells us also how the ultimate elements of the nucleus, fibrils most likely, are first duplicated and then separated. It is in this regard that the performance of the standard fixation still falls short of the ideal, at least where it has been most tested, namely in the bacterium *E. coli*. The extraordinary sameness of all the nucleoplasms in all the cells of a given sample, the lack of differences between the fine structure of nucleoplasms from growing and from starving bacteria, and the frequently complete randomness of their constituent fibrils make it hard to think of means for their orderly multiplication and segregation. Aware of this puzzle Ryter and Kellenberger (1958) state that: "La forme de la vacuole nucléaire n'a pas de signification fonctionelle importante; elle est simplement le récipient du nucléoplasme composé lui même de fibrilles d'ADN ou de desoxyribonucléoprotéine. C'est donc au niveau de ces fibrilles qu'il faut rechercher le mécanisme de réplication. . . ."[1] One can agree with this conclusion without denying significance to the *shape* of the nucleus. It is true that the configuration of bacterial chromatin is liable to many changes of shape not arising from its division and that its configuration is often complicated beyond hope of immediate understanding, but we know also that in certain favorable bacteria the nuclei are units with constant properties and that in these species, under certain conditions of cultivation, the configuration and optical properties of the living nucleus (and hence presumably its ultrastructure) vary in a regular and predictable manner with the phases of its division. In other words, here each phase of division does have its own constant and recognizable shape. The electron microscopy of this type of neat and intelligible bacterial nucleus is an urgent and promising task. Attempts should be made to recognize the phases of its division in micrographs of longitudinal sections, and correlated changes in the number, thickness, and arrangement of the nuclear fibrils should be looked for.

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[1] "The shape of the nuclear vacuole has no important functional significance; the vacuole is merely the recipient of the nucleoplasm, itself composed of DNA fibrils or of deoxyribonucleoproteins. Thus, it is at the level of these fibrils that one must look for the mechanism of replication. . . ."

Bundles of either paired or single fibers that follow the same course over considerable distances have occasionally been seen in rod-shaped bacteria fixed by the standard fixation, particularly clearly in a section of *Salmonella typhimurium* (Birch-Andersen and Maaløe quoted in Ryter and Kellenberger, 1958) and in a cell of *E. coli* kept starving in buffered saline for 1 hour (Kellenberger *et al.*, 1958). Orderly arrangements of fibrils, reflecting perhaps division movements, are regularly seen in the nuclei of two different species of cocci now being studied in the laboratory of Dr. Woutera van Iterson at Amsterdam. Nuclei of one of them, the tetrad-forming species of Figs. 1 and 19, are illustrated in Fig. 25.

C. Some Current Problems

There are many wide lacunae in our knowledge of bacterial nuclei. Their chemistry is imperfectly known, their low affinities for simple basic stains and their response to hydrolysis with $N/1$ HCl at 60° C. have not yet been properly explained. In electron micrographs the special protein core on which their chromatin is said to be wound and which is set free together with the nuclei during the lipase-mediated disintegration of free protoplasts (Spiegelman *et al.*, 1958) is indistinguishable from enclaves of cytoplasm in the nuclear vacuole.

Most puzzling is the ability of the nuclei to lose their identity and to link up with their fellows in the same cell to form a continuum and some time later to emerge from it once more and resume a separate existence. This happens spontaneously during spore formation (Young and Fitz–James, 1959a), and such a course of events can be started with ultraviolet light (Kellenberger, 1953), changes in the concentration of NaCl around the bacteria (Whitfield and Murray, 1954), chilling and sublethal doses of tetracylines (Whitfield and Murray, 1954; Kellenberger,

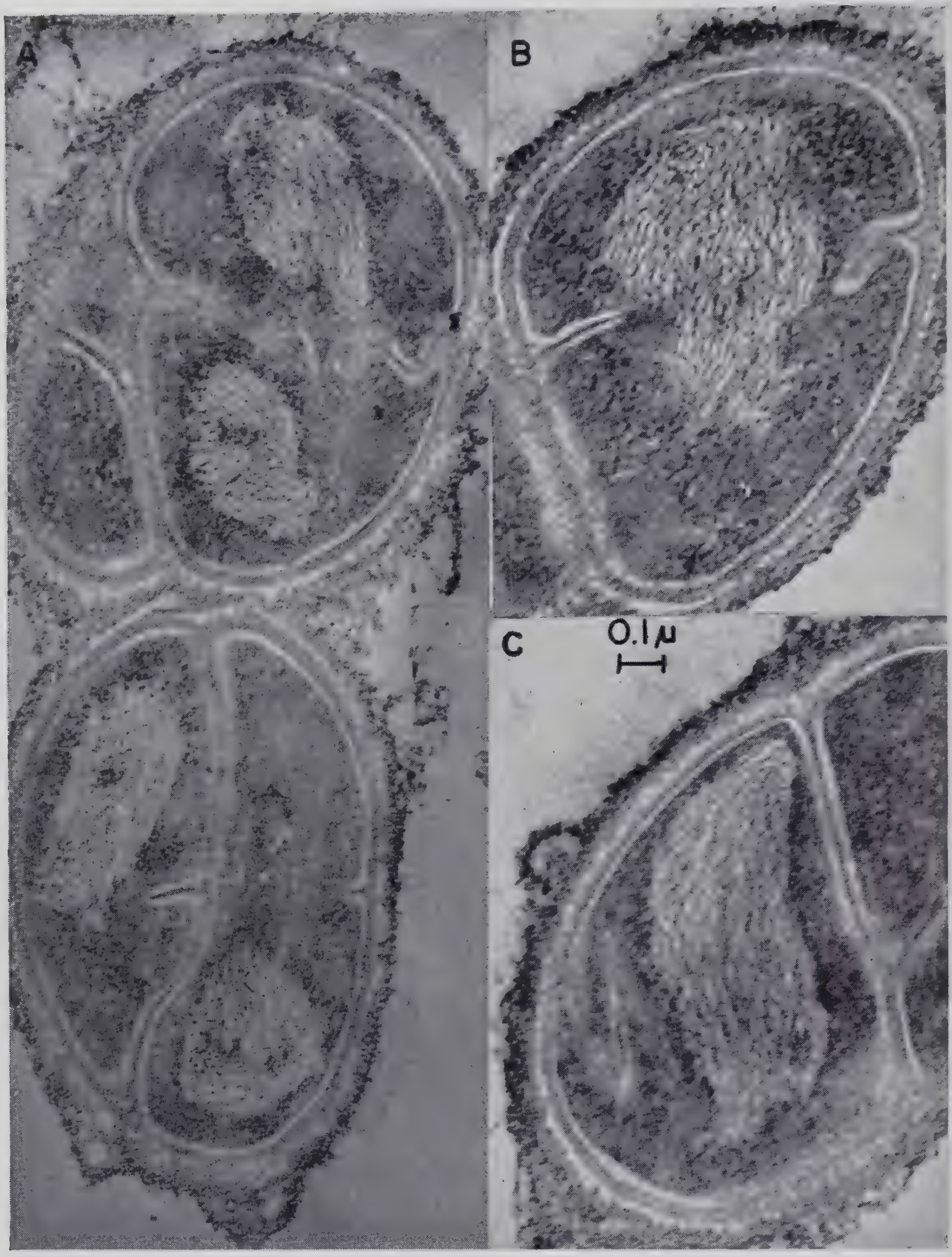

Fig. 25. Electron micrographs of sections of the tetrad coccus of Figs. 1 and 19 prepared by Dr. Woutera van Iterson. Fixed and embedded according to Kellenberger. The nuclei contain numerous fine fibers. Those in B and C look as if they have been dividing at the time of fixation.

1953). The ability of bacterial chromatin to behave at one time as a unit and at another time to submerge itself in a continuum places bacterial nuclei, as regards chromosome topology, into a class with the macronuclei of ciliates and the nuclei of spermatoza. Macronuclei contain many sets of chromosomes, perhaps in the form of many identical chainlike replicas of the whole genome or haploid set of chromosomes (Grell, 1952). The fibrils of bacterial nuclei may similarly represent identical replicas of the single linkage group which is the working unit of bacterial geneticists (Kellenberger *et al.*, 1958). The purely formal analogy with the nuclei of spermatozoa consists in this, that in the continuum type (or phase) of bacterial nuclei the endowments of several nuclei are inextricably mixed in a way which yet preserves their identity. So too in many types of sperms the component fibrils of separate chromosomes are bundled together into a structure of such crystalline uniformity that the boundaries of chromosomal domains become for a time invisible (Gall and Bjork, 1958).

Information about the mode of division of bacterial nuclei can also be obtained in an indirect manner, namely, by determining the rate of synthesis of DNA in a culture of synchronously dividing bacteria. If the nucleus is a continuum whose elements duplicate themselves independently of each other, then new DNA should appear at a constant, steady rate. But if the nucleus is a unit package containing a fixed number of elements, all of which grow, duplicate, and segregate at the *same time* and rest for an appreciable period before repeating this cycle, then increases of DNA should follow each other in a series of discrete steps. It is puzzling at first to learn that *both* kinds of rates of increase of DNA have been observed by different workers (Maruyama and Yanajita, 1956; Burns, 1959; Barner and Cohen, 1956; Schaechter *et al.*, 1959) and that those cultures which have been synchronized in the most natural manner, namely, by heat-activating a batch of spores and seeding them into a medium of optimal composition, always give smooth curves of DNA increase, with only a slight slowing down during cell division (Young and Fitz–James, 1959b). This is close to what one would expect to find if sister nuclei were to start duplicating themselves at the very moment of their emergence from the previous division. The behavior of the nuclei of *B. cereus* var. *mycoides* in Fig. 23 is an example of this. It would be interesting to see whether DNA increases stepwise in cultures of *B. subtilis*, whose nuclei seem to go through a short phase of rest between divisions.

D. Membrane Systems in Contact with Nuclei

The nuclei of *B. megaterium*, as DeLamater and Mudd (1951) and DeLamater (1953b) have shown and others have confirmed (Robinow,

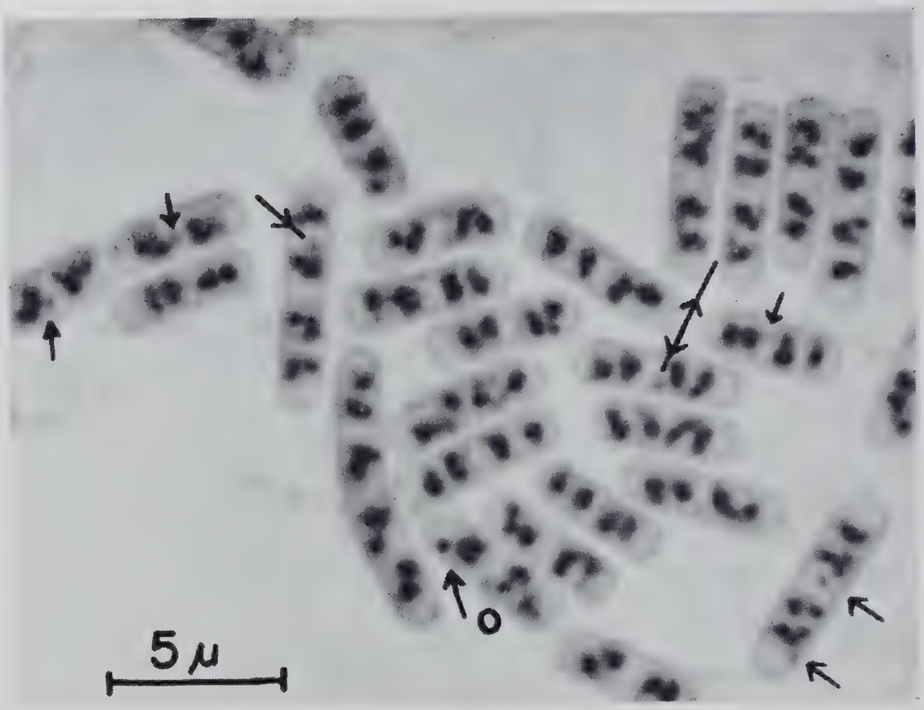

FIG. 26. *Bacillus cereus,* grown on heat infusion agar at 37 C., OsO_4, HCl, Giesma. Arrows point to accessory chromatin granules—a dubious one is marked with a circle. Compare with Figs. 18 and 24 of the same strain. Magnification: ×3600.

1956b), are accompanied closely or at a slight distance by one or two small round bodies which have been called "centrioles" and also "accessory chromatin granules." In hydrolyzed preparations they retain the azure A-SO_2 or thionine-SO_2 stains after long dehydration with alcohol. They are also clearly visible in HCl-Giemsa preparations (Figs. 26, 27, A) and objects resembling them are seen in Feulgen preparations (Fig. 27, B). Some of DeLamater's illustrations suggest that the small bodies are capable of growth and division. According to DeLamater the satellite bodies are centrioles, but electron microscopy has provided no evidence of the existence in bacteria of bodies having the now well-known fine structure of centrioles (Bernhard and de Harven, 1956). In fact, until the recent introduction of Kellenberger's methods of fixation and embedding in "Vestopal" polyester (Ryter and Kellenberger, 1958), nothing resembling the problematic bodies seems to have been noticed in electron micrographs of *Bacillus* cells. Recently, however, round complexes of concentric double-contoured membranes (Fig. 28) have been discovered by Dr. W. van Iterson in contact with the nuclei of the same strain of *B. cereus* shown in Figs. 26 and 27. Their size and shape and their closeness to the nuclei suggest that the concentric membrane systems correspond to the accessory chromatin granules of stained preparations. In another laboratory, using the same methods, elaborate systems of this kind have

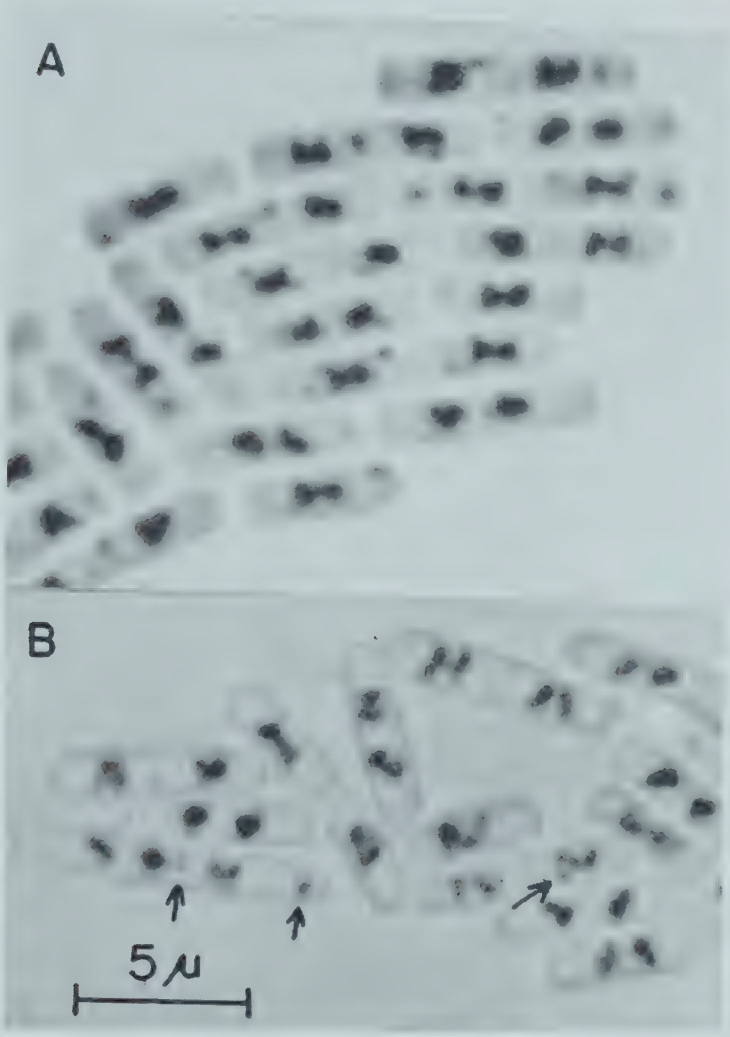

FIG 27. *Bacillus cereus* fixed 10 minutes after transfer from a low- to a high-salt medium (see text for details). In A, contraction of the nuclei has almost everywhere revealed the presence of the small accessory granules. OsO_4, HCl, Giemsa. Preparation and micrograph by Dr. R. G. E. Murray. B. A less successful repetition of the same experiment. OsO_4, Feulgen, no counterstain. Arrows point to accessory granules. Only small numbers were visible in both Giemsa and Feulgen preparations. Some, presumably, are hidden among the contracted nuclei.

at the same time been discovered in *Streptomyces coelicolor* (Glauert and Hopwood, 1959). These too are often close to, or partly surrounded by, the nuclei. Elsewhere they are continuous with the plasma membrane. The whirls of concentric lamellae bear some resemblance to mitochondria. Some profiles of them look not unlike a fragment of an endoplasmic reticulum (Fig. 28, B). The fact, true for *B. cereus* at any rate, that these organelles remain basiphilic after hydrolysis does not go well with either of these identifications, but it is too early to do more than draw attention to the existence of these hitherto unsuspected structures. A fine specimen

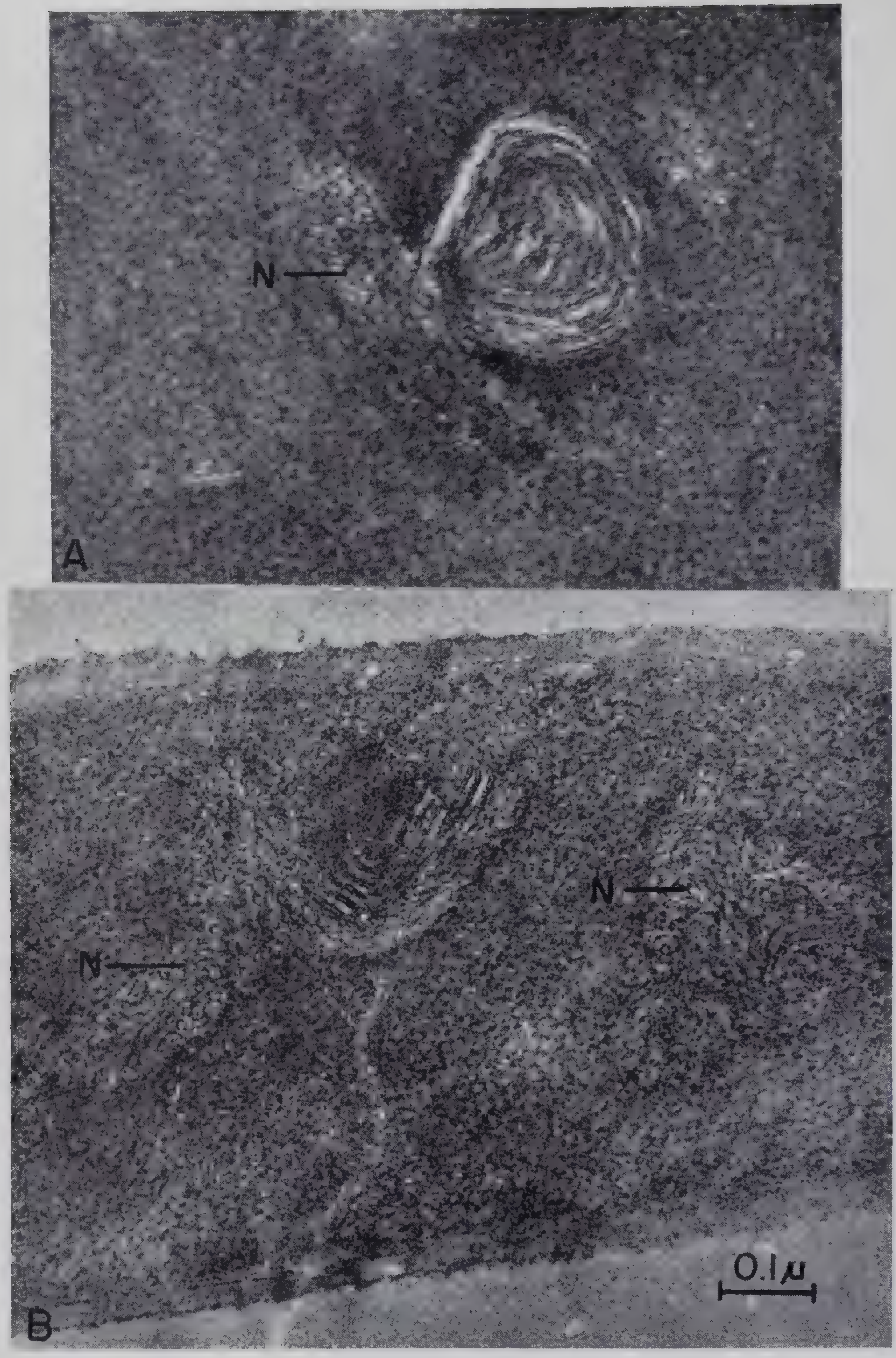

Fig. 28. Electron micrographs of sections of *Bacillus cereus* from a culture comparable to that which provided the preparation illustrated in Fig. 26. In both A and B the nucleus (*N*) is in contact with a system of membranes believed to correspond to an accessory chromatin granule or "centriole." Micrographs prepared by Dr. Woutera van Iterson.

of a lamellated body is to be seen in a micrograph of a section of *B. subtilis* published by Ryter and Kellenberger (1958), and it seems possible that the solitary dense, faintly structured round bodies in *E. coli* described by both Kellenberger *et al.* (1958) and Niklowitz (1958) and regarded by them as mitochondrial equivalents, are of the same nature.

XI. SPORES

A. *General Description* (*Fig. 29*)

Some bacteria are capable of interrupting their cycle of growth and multiplication with a state of complete rest which is at the same time one of greatly increased powers of resistance to dryness, heat, and many other damaging influences. They do this by developing inside their cytoplasm a special kind of cell which is supplied with a nucleus, deprived of almost all water, surrounded by several membranes differing in composition, and eventually set free. Bacterial cells in this condition are called spores or endospores, and their presence everywhere has compelled surgeons and those concerned with the preservation of foods and other perishable materials to develop efficient methods for their destruction. Quite apart from these practical matters, the properties of a dormant passive cell which can be aroused to renewed activity at will in a few minutes in media of known composition are of interest to biologists in general (Knaysi, 1948; Halvorson, 1957; Symposium edited by Jacobs and Clegg, 1957; Robinow, 1960).

Bacteria genetically endowed to do so form spores when the environment becomes depleted of one or more factors essential for growth, provided various metals and minerals are present at the same time in adequate and balanced amounts (Curran, 1957). Potassium, for example, is essential for spore formation by *B. cereus* (Foster and Heiligman, 1949a, b); no spores are formed by *B. subtilis* unless minute amounts of manganese are available in the medium (Charney *et al.*, 1951); potassium, magnesium, calcium, and manganese are required for spore formation by *B. megaterium* (Grelet, 1952a, b); and *B. coagulans* forms few spores unless supplied with inorganic sulfur (Ordal, 1957). In media designed with these facts in mind it is feasible to induce all cells to form spores at the same time and reap harvests of 10^8 spores per milliliter (Young and Fitz–James, 1959a).

Spores differ from the cells in which they arise in being very dry (Murrell and Scott, 1957), in having about ten times as much calcium as vegetative forms (Curran *et al.*, 1943), and in containing relatively large amounts (3–15% of the dry weight) of the calcium salt of dipicolinic acid (pyridine-2, 6-/dicarboxylic acid, DPA) a substance which was discovered

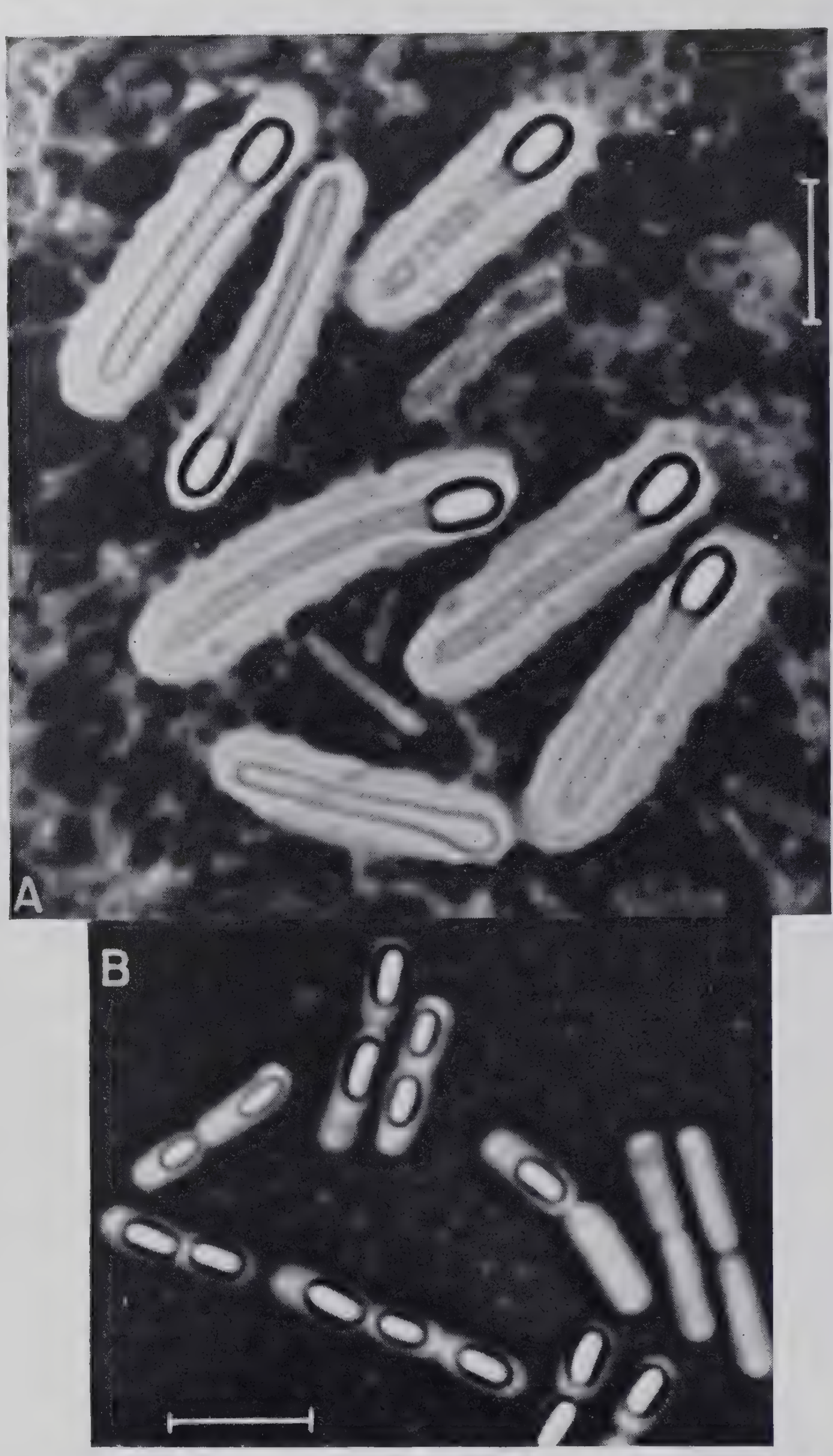

Fig. 29. Bacteria forming spores. A. *Clostridium pectinovorum.* Four days. Anaerobic culture. The spores are the shiny ovals at one end of the long, capsulated cells. When they have matured they will be set free and the remains of the vegetative cell and the capsule will disintegrate. Near the lower margin is a cell whose swollen tip shows that it has begun to form a spore. The vegetative form of this organism is a straight, slender rod without a capsule. A few are seen in the background. Compare with Fig. 8, which illustrates an earlier stage of spore formation, and with Fig. 32, which shows the behavior of the chromatin during this process. B. Spore formation in *Bacillus cereus;* 18 hours; potato agar. Both A and B are from nigrosin films on coverslips mounted on slides, with the bacteria facing downward into air. The photographs were taken with an immersion lens through the clean glass of the back of the coverslip. The scales represent 5 μ.

in spores by Joan Powell and which is absent from vegetative cells (Powell, 1953).

The basis of the hardiness of spores is not precisely known, but there is evidence that their heat resistance is related to their content of calcium and DPA (Church and Halvorson, 1959). It has been suggested that DPA not only heat-proofs the protoplasm but assists its reawakening by chelating metal ions which would otherwise inhibit energy-giving oxidizing reactions (Halvorson *et al.*, 1958).

Spores of different species differ from each other on the outside. Some are smooth, others are intricately sculptured (Franklin and Bradley, 1957; Robinow, 1960). They differ also in the number of envelopes which surround the dormant bacterium in their interior. Spores of *B. subtilis* have a coat of many different layers (Tokuyasu and Yamada, 1959b), those of *B. cereus* a simpler one. At the present time, when techniques are continuously being improved, the number of envelopes that can be distinguished in electron micrographs varies not only with the species, but also with the method that has been used in preparing the spores, and it will be some time before all workers in the field are agreed on the details of spore structure.

It is now generally agreed that there are two regions in the interior of spores: a kernel of dense protoplasm, and a rind or *cortex* of less dense material (Fig. 30) (Mayall and Robinow, 1957). The inner region is the dormant bacillus. It is strongly basophilic (at least after slices of it have been in contact with water) and contains near its surface a few branching cords of Feulgen-positive matter, the nucleus, which in electron micrographs appear as vague shapes of very low density. The cortex has no affinity for stains and as little or less scattering power for electrons as the nucleus. Its composition is not known, but the example of glycogen and of its bacterial equivalent "granulose," both of which appear "white" in electron micrographs, makes it likely that the cortex contains some polysaccharide and little or no phosphorus. In sections cut from osmium-fixed *B. megaterium* spores that had been soaked for 20–30 minutes in $N/1$ HNO_3 at room temperature, the cortex appears fibrous and laminated (Mayall and Robinow, 1957). Layering of the cortex has also been seen, without recourse to acid, in developing spores of *B. cereus* and *Clostridium pectinovorum* (Young and Fitz–James, 1959b; Murray, 1958). During the germination of spores of *B. megaterium*, the only species examined in this respect, the cortex first breaks down into a loose, more or less randomly orientated spongework of fibers and soon disappears altogether. At its inner surface appears at the same time the new cell wall, nourished perhaps by materials made available by the disintegrating cortex. A spore fixed at an early stage of germination with much of the cortex still left

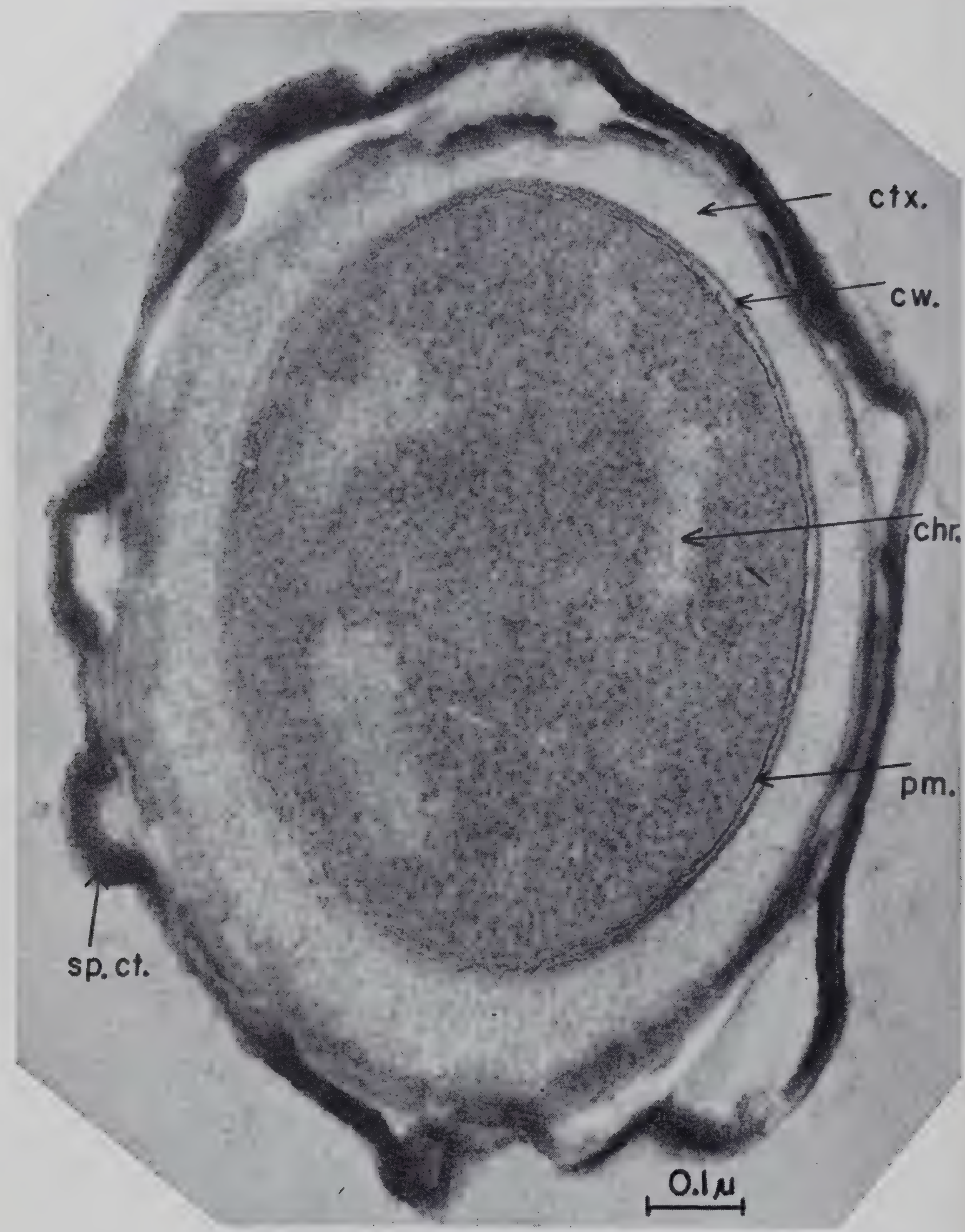

FIG. 30. Section through a germinating spore of *Bacillus megaterium* which is still inside its envelopes and has retained some of the features of the phase of rest. The outer coat (*sp. ct.*) (two can usually be distinguished in *B. megaterium*) is not well preserved. The cortex (*ctx.*) has a loose, spongy structure. It is in the process of being dissolved. The cell wall (*cw.*) is still very thin; it will soon become thicker. The plasma membrane (*pm.*) is very dense and has the many irregularities of outline which (after the fixation here employed) it has in fully developed vegetative cells. The chromatin (*chr.*) is featureless and lacks the distinct fibrils which seem to compose it in mature vegetative forms. The cytoplasm is coarse grained and already typical of that of a growing bacterium. In a resting spore it is much finer and denser. Fixation and embedding after Kellenberger. Electron micrograph by Dr. G. de Haller.

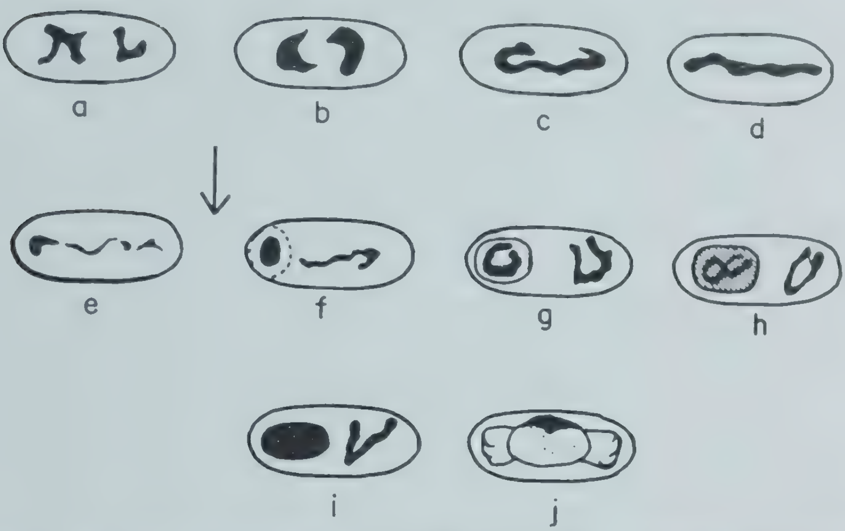

FIG. 31. Diagram illustrating the behavior of the chromatin during spore formation in hydrolyzed and stained preparations of *Bacillus cereus*. The arrow indicates the point in time where the spore first becomes visible in the living cell; a–h based on Young and Fitz-James. In i the spore has become so basophilic all over that staining now obscures the chromatin in its interior. In j the chromatin is shown extruded to the periphery in response to acid hydrolysis. The spore is shown already inside its special investment or exosporium, but still within its mother bacterium. The spores in this diagram correspond to those in Fig. 9, B. Free spores of the closely related variant *B. cereus* var. *mycoides* are shown in Fig. 33.

in place is illustrated in Fig. 30. In several species a delicate precursor of the cell wall is visible already during the resting stage. It used to be thought that all parts of the spore arise new in the cytoplasm, but Young and Fitz–James have made the surprising discovery that in *B. cereus* and several related forms the delicate cell wall anlage or first "spore wall" at first grows out from the inner surface of the existing, much thicker wall of the mother bacillus and only later detaches itself from its place of origin and comes to lie free in the cytoplasm (Young and Fitz–James, 1959a). Traces of a similar process have already been seen in *B. subtilis* (Tokuyasu and Yamada, 1959b), and it seems that in several species spore formation will now have to be regarded as a special form of cell division and no longer as an example of free cell formation.

B. Nuclear Changes During Spore Development (Figs. 31, 32)

When cells of *Bacillus* species begin to form spores they stop growing. At that time they usually contain two nuclei. Recent observations on preparations from synchronized sporing cultures of *B. cereus* have established, with greater precision than had been attained by earlier students of the same event, that the two nuclei of a vegetative cell about to form a spore, first thicken and then stretch out in the long axis of the bacillus

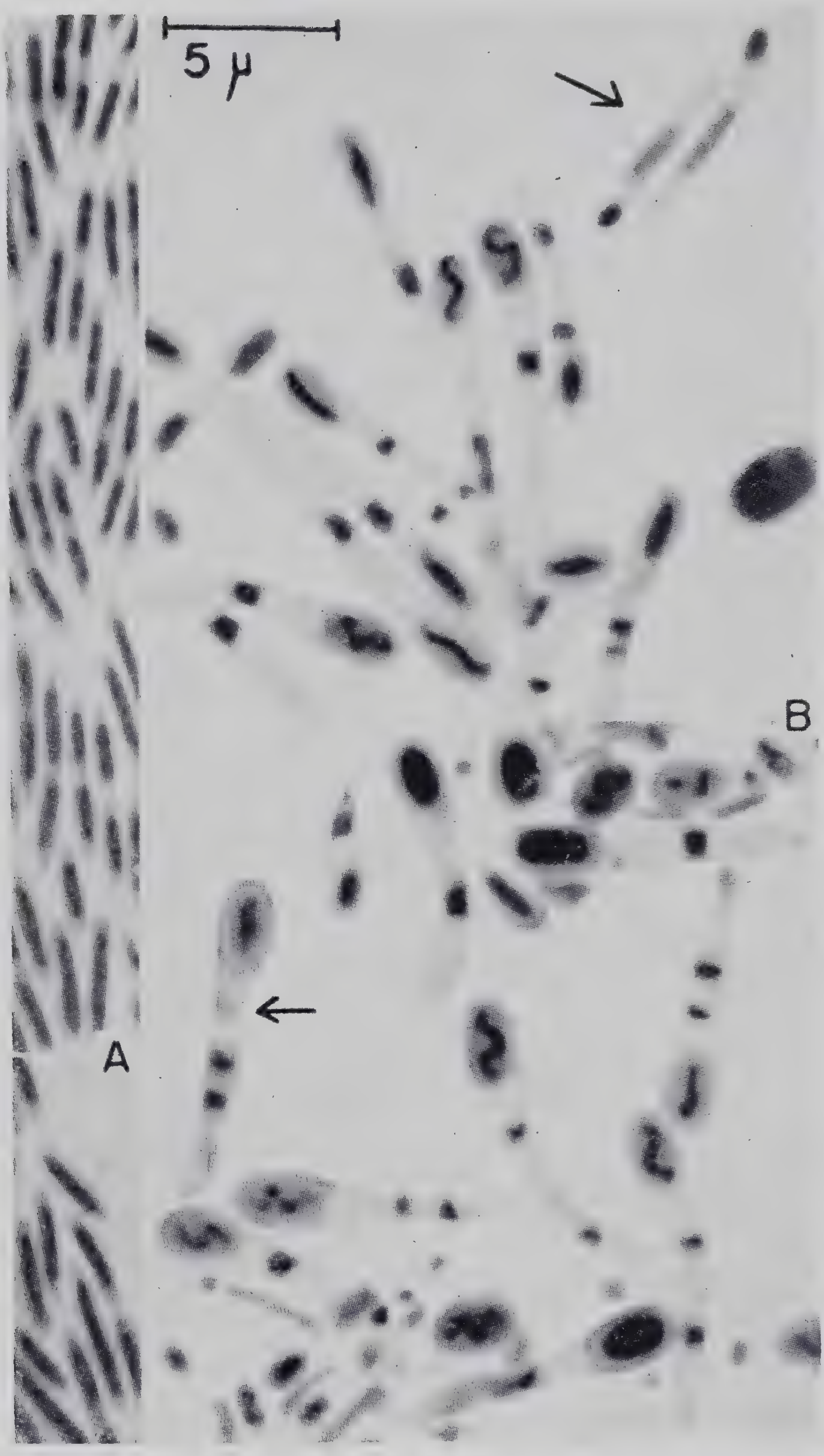

FIG. 32. Behavior of the chromatin during spore formation in *Clostridium pectinovorum* (see Figs. 8, B and 29, A). A. Tightly packed columns of minute nuclei (or, perhaps, one long continuous one ?) in the slender rods of the vegetative phase. B. Top right: early stage of segregation of some of the chromatin to one end of the cell. Elsewhere many examples of the transformation of the spore chromatin into first a straight, and later a helically twisted, filament. Nearly matured spores are basophilic all over, and the heavy staining which they accept masks the coiled nucleus within. The nucleus emerges into view again, still coiled, at the onset of germination. Acetic acid-alcohol, HCl, Giemsa.

and for a time fuse into an axial cord of chromatin (Young and Fitz–James, 1959a). Later this breaks up again into two nuclei, and around one of these the spore is constructed. Whether these nuclear manoeuvers achieve something of genetic importance for the spore is at present unknown. The fleeting appearance of fusion nuclei during spore formation is common, but it is not certain that the nuclei of all sporing forms behave in the same way. In *Clostridium pectinovorum*, in which each vegetative cell contains many small nuclei, it seems that the one that is nearest to the sporing end of the cell is directly delegated "for spore duty" (Fig. 32) (Robinow, 1960). However, the narrowness of the cells of this organism makes it difficult to obtain a clear picture of the nuclear events during early stages of spore formation.

Attempts to stain the nucleus of the mature spore encounter difficulties. One cannot look into living spores. Stains do not penetrate to their interior, and exposure to acid, which is an essential part of the Feulgen procedure for the detection of DNA, inevitably induces a serious topographical artifact, namely the extrusion of the chromatin through a gap in the ruptured spore coat (Bisset and Hale, 1951; Robinow, 1951). It is therefore necessary to crack spores open or section them before their chromatin can be examined *in situ*. Both techniques have been used (Fitz–James, 1953; Robinow, 1953) and have shown that the spore's chromatin is close to the surface of the inner core of cytoplasm. Three-dimensional reconstructions have not been made, but in first approximation it may be said that, in spores of *B. megatherium* and *B. cereus* at any rate, the chromatin appears to be in the shape of a spherical net consisting of a few branching cords, rather like a practice golf ball with three or four large perforations. The nucleus of spores of *C. pectinovorum* retains through the period of rest the shape of a loosely coiled helix which it acquired during the development of the spore (Robinow, 1960).

C. *Spore Germination*

In a medium of suitable composition—the presence of L-alanine is often important here (Hills, 1949)—a spore swells, bursts, sheds its coat, and changes into a new vegetative bacillus (Fig. 33). Its cytoplasm, featureless as ground glass in electron micrographs of spores fixed at rest, now becomes granular. The nuclei, scarcely detectable in the resting spore, turn into distinct "nuclear vacuoles." A cell wall is built and the cortex is lost.

The chemistry of germination has been studied intensely of late (Powell, 1953; Halvorson and Church, 1957) and has led to the discovery that spores secrete solid matter corresponding to 30% of their dry

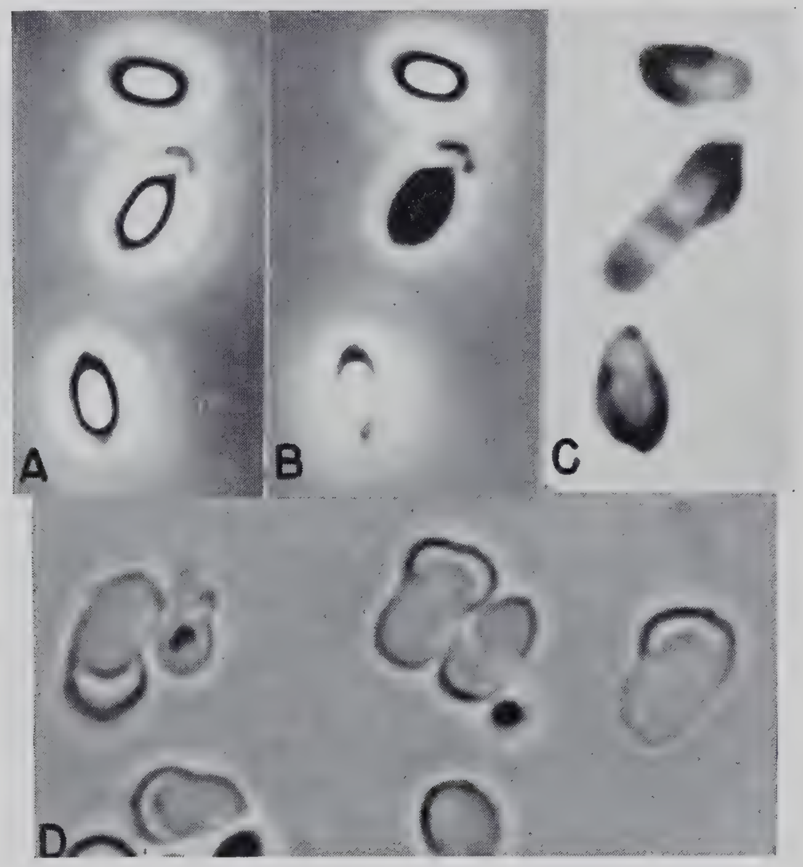

Fig. 33. Germinating spores. A–C. Time lapse, phase contrast micrographs of stages in the germination of three spores of *Bacillus cereus* var. *mycoides*. Resting spores (A) are very dense and shine brightly. Much of the refractility is lost when germination begins. This has happened in the central spore in B. Growing bacteria are emerging from ruptured spore cases in C. The two white shapes in the central bacterium are images of its nuclei (see Fig. 23). B. Young vegetative cells emerging from germinating spores of *B. megaterium*. Living slide culture, ordinary microscopy. A and C. Magnification: ×3800. B. Magnification: ×3600.

weight into the medium very early in germination (Powell and Strange, 1953).

Concentric arrays of double-contoured membranes are not obvious in resting spores but they appear early in germination in the cytoplasm of the young vegetative cell (Fig. 34).

XII. Mechanisms of Gene Transfer

(Jacob, 1959; Lederberg, 1959; Hartman and Goodgal, 1959)

The discovery of gene recombination in bacteria by Tatum and Lederberg in 1947 (Lederberg, 1947) has initiated a large volume of work on bacterial genetics. Three mechanisms of gene transfer are now recognized: transformation, transduction, and conjugation. All three were found and are being studied in laboratories. It is uncertain to what extent

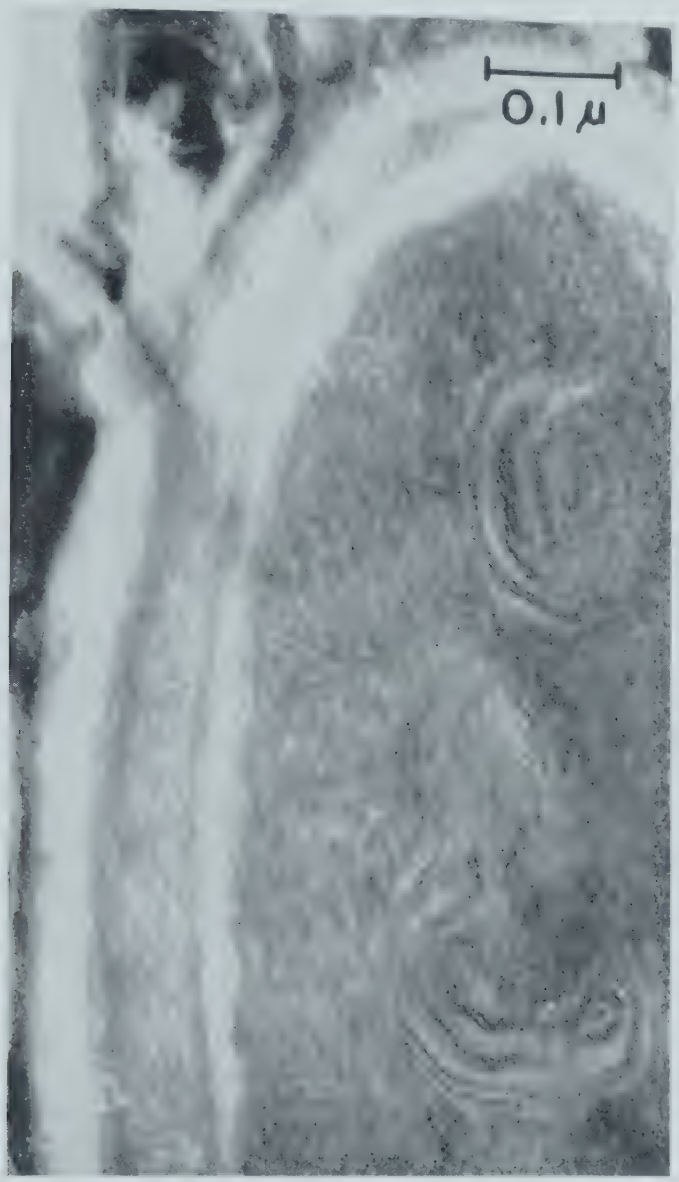

FIG. 34. Two complexes of concentric membranes, about 40–60 A. thick, in the cytoplasm of a germinating spore of *Bacillus megaterium*. Compare with Fig. 28 of *B. cereus*. Electron micrograph by Dr. G. de Haller.

they are employed by bacteria living in a natural environment. Meanwhile the analysis of these phenomena has contributed much to the rising science of genetic chemistry.

A. *Transformation*

A bacterium is said to have been transformed if absorption of DNA prepared from a related donor causes properties of the donor to appear in the recipient and its offspring. Characters that have been conferred in this way include resistance to penicillin and/or streptomycin and the ability to synthesize the polysaccharides of capsules. The physiology of transformation has been thoroughly explored by Hotchkiss (1956) and by Ephrussi-Taylor (1955). First achieved in pneumococci, transformation has also been demonstrated in influenza bacilli (Alexander and Leidy, 1953) and in *B. subtilis* (Spizizen, 1958).

B. Transduction (Zinder and Lederberg, 1952)

This feat is accomplished by a temperate, that is to say a more or less tolerated bacteriophage which is capable of transmitting to a fresh host one or two hereditary traits of its last victim. Transduction has been mainly studied in *Salmonella.* A superficially similar but genetically different effect, *lysogenic conversion,* has been discovered in avirulent diphtheria bacilli on which infection with a certain temperate phage confers the ability to make toxin (Freeman, 1951). Manufacture of toxin ceases as soon as the phage is lost from the cell; it is thus under the control of the phage and not, as in transduction proper, a newly and permanently acquired part of the hosts' genome (Groman, 1955).

C. Conjugation

This is the temporary union of two whole living bacteria followed by a reshuffling of the genetic endowment of one of them. It has been observed in certain strains of *E. coli* and in mixtures of *E. coli* with *Shigella* (Luria and Burrous, 1957) and also occurs in the cholera vibrio (Bhaskaran, 1958) and in *Pseudomonas* (Holloway and Jennings, 1958). Conjugating bacteria are linked for an hour or so by a bridge of protoplasm which serves to transfer part of the genome of a male cell to a female one. The transfer is in one direction only, but an exchange of genes between paternal and maternal chromosomes takes place in the zygote. Originally a rare event occuring once in a million of unfertile contacts, conjugation can now be studied in certain mutant strains where it can be induced to take place with a frequency of 10% or more. Anderson (1958), who has studied the conjugation of members of two visibly different strains distinguishes three phases:

1. Tentative contact with invisible fibers connecting the prospective mates.
2. Copulation, involving close joining of the two bacteria, end to end or end to side.
3. Separation of the apparently intact bacteria.

There is both genetical and chemical evidence that during conjugation something is transferred in one direction from the donor bacterium to the receiving one. The transfer of the paternal genome can be stopped by shearing copulating partners apart in a high-speed blender. The shearing experiments have revealed that fertilization is progressive and that the mutant markers gliding across from the donor, already known from conventional genetic experiments to be members of a single linkage group, are arranged linearly and in constant order (Jacob and Wollman, 1958). An exception is the fertility or male competence factor F which is transferred independently of the linked factors and is regarded as a cytoplasmic particle.

Chemical analysis of the DNA of zygotes in which the male contribution came from bacteria whose DNA had been labeled with P^{32} has shown that DNA is transferred during conjugation (Garen and Skaar, 1958). The amount transferred corresponds to about 10% of that present in the donor bacterium at the beginning of the experiment. It is not known in what form this DNA is transferred. The genetic terms "chromosome" and "nucleus" have been tacitly widened to include the stainable, visible chromatin-containing organelles of bacteria. But it has not yet been shown that the stainable nucleus of *E. coli* takes part in conjugation (Lederberg, 1956). The 10% of paternal DNA that goes into the zygote may represent a small fraction of one visible nucleus, "nucleoid," or "nucleoplasm" or it may come from a different, as yet unrecognized, source. An uncoiled single thread of DNA or DNA protein, as which the bacterial chromosome is now regarded, would in any case be invisible.

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NOTE ADDED IN PROOF

Recent information on the nucleus and the genetics of bacteria will be found in "Microbial Genetics" (W. Hayes and R. C. Clowes, eds.) *10th Symposium, Soc. Gen. Microbiol.* (*Cambridge, Engl.*) 1960.

CHAPTER 3

Protozoa

By DAVID L. NANNEY and MARIA A. RUDZINSKA[1]

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I. INTRODUCTION

Protozoan "cells" may be considered "generalized" animal cells only in a restricted sense; they are limited in their capacity to specialize by their separate existence. Each cell is a complete organism and must carry out all the functions required for life without the economically useful division of labor employed by cells in higher organisms. However, even cells in higher organisms are not capable of partitioning all cellular functions, and liver, nerve and muscle cells share many common biological properties. Moreover, the number of diverse habitats populated with different protozoan species permits a vast variety of structural and functional modifications. Tens of thousands of species of protozoa occur; some live in the soil; others are free-living in either salt- or fresh-water environments; still others are parasitic or symbiotic and occupy countless habitats within or upon other organisms. Protozoa vary from sizes near the limits of optical microscopy to sizes visible with the naked eye. Morphologically they vary from the relatively unstructured amebas to the highly differentiated oligotrichous ciliates of cattle, with their complex locomotor and skeletal organelles and their specialized nuclear apparatus. Physiological variations are not likely to be less dramatic. Even within a single species of protozoa, particularly among the parasitic forms, striking diversities in size, physiology, and morphology can be observed.

The structural and functional variety within the protozoa makes impossible any generalized synthetic treatment of "the protozoan cell" in a work of this scope; if the protozoa are to be considered as cells, much of the science of protozoology is concerned with cellular structure, function, and biochemistry. Instead we will have to focus attention on one group of protozoans and limit our discussion to problems whose analysis is especially facilitated by their peculiar biological properties. In part because of their unique characteristics—which permit the asking of unusual kinds of questions—and in part because of the large amount of study devoted to them, the ciliates have been chosen for this presentation. Even for this group the coverage will be limited and will center on problems of cellular integration.

II. CELL STRUCTURES AND THEIR INTERRELATIONS

The ciliated protozoa are characterized by two special structural features which set them apart and permit experimental analyses of a sort not readily undertaken in other forms; they have a highly complex visible pellicular and subpellicular organization, and they have a unique nuclear apparatus involving two kinds of nuclei which undergo striking alterations at certain times in the life cycle.

A. Cortical Organization

1. Kinetosomes

The smallest elements visible by light microscopy which contribute to the cortical structure in ciliates are argentophilic granules (Fig. 1) designated as *kinetosomes* (Figs. 2, 3, 5) (see Chatton and Lwoff, 1935; Fauré-Fremiet, 1948; Lwoff, 1950). They are related in structure and

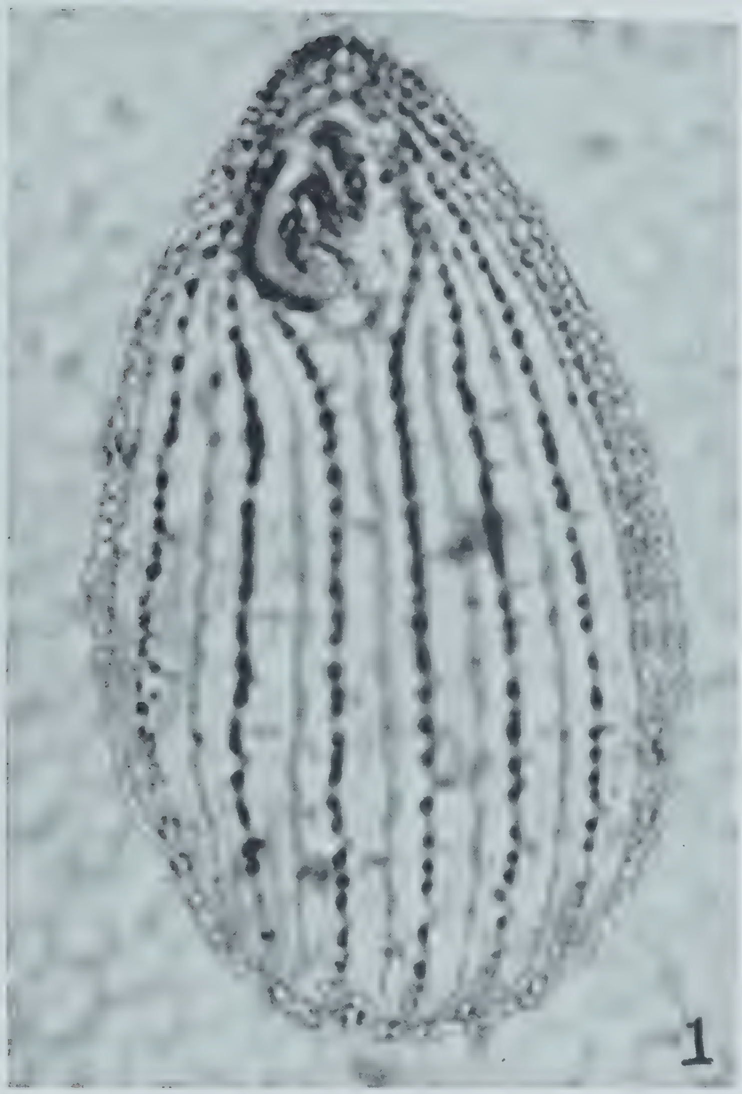


FIG. 1. *Tetrahymena pyriformis,* mating type I of variety 1. Ventral view of specimen impregnated with silver according to the Chatton-Lwoff technique. Note the rows of argentophilic basal granules or kinetosomes and the infraciliary bases of the tetrahymenal buccal apparatus. The curved black line on the right (viewer's left) represents the base of the *UM* (undulating membrane). The line of kinetosomes leading posteriorly from the posterior end of the *UM* is known as the stomatogenous kinety or meridian since it is responsible for production for the new buccal apparatus acquired by the posterior daughter in binary fission. The cytoproct or cell anus occurs near its posterior end. Cilia are not visible in this silver preparation; neither are the kinetodesmata. Magnification: approximately ×1700. Photomicrograph kindly supplied by Dr. John O. Corliss, University of Illinois.

function, and possibly in origin, to granules associated with kinetic elements (cilia, flagella, spindles) throughout the plant and animal kingdoms. Their primary function appears to be that of giving rise to the cilia (Figs. 5, 9), but at certain times in the life cycles of some ciliates this function may be suppressed (Figs. 2, 3). In certain species they seem

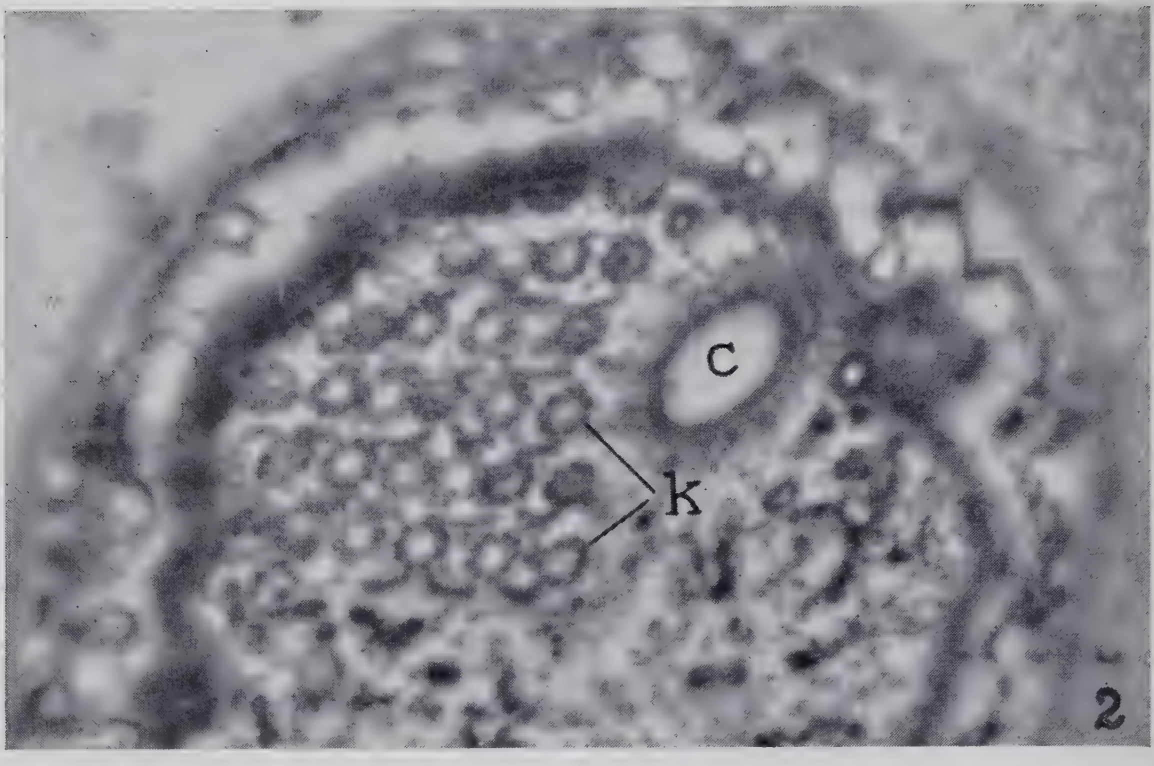


FIG. 2. Electron micrograph of oblique section cut close to the surface of an adult *Tokophyra infusionum*. Six rows of kinetosomes (basal bodies) (*k*) in cross section are seen on one side of the canal (*c*) of the contractile vacuole. The adult *Tokophyra* is sessile and does not possess cilia. In the life cycle of *Tokophyra* there is, however, a motile stage, a so-called embryo with cilia. The cilia disappear during metamorphosis, but the kinetosomes (*k*) as indicated in the electron micrograph remain in the adult stage and give rise to cilia in the developing embryo. Magnification: ×24,000. (Rudzinska, 1958.)

to be responsible for the production of the extrusible *trichocysts* (Figs. 4, 25) and *trichites* (Lwoff, 1950). Associated with them and perhaps produced by them are fibers, *kinetodesmata* (Fig. 5), which contribute to the subpellicular network of fibers (Metz *et al.*, 1953). The kinetosomes also appear to serve as "organization centers" responsible for the geometric regularity of the pellicle surface. Most important, the kinetosomes appear to give rise to more kinetosomes through a process of division (Lwoff, 1950). However, little is certainly known at the present time of the origin of new kinetosomes, of their structures, or of their functions; a full evaluation of their significance must await further studies, particularly those employing electron microscopy. Nevertheless, because they appear to divide, the kinetosomes have been considered to be cytoplasmic genetic elements, having both an autocatalytic function (forming new kinetosomes) and one or more heterocatalytic functions (forming cilia, trichocysts, trichites, and fibers). Kinetosomes are usually considered pluripotent: a single granule may after a few divisions yield daughter

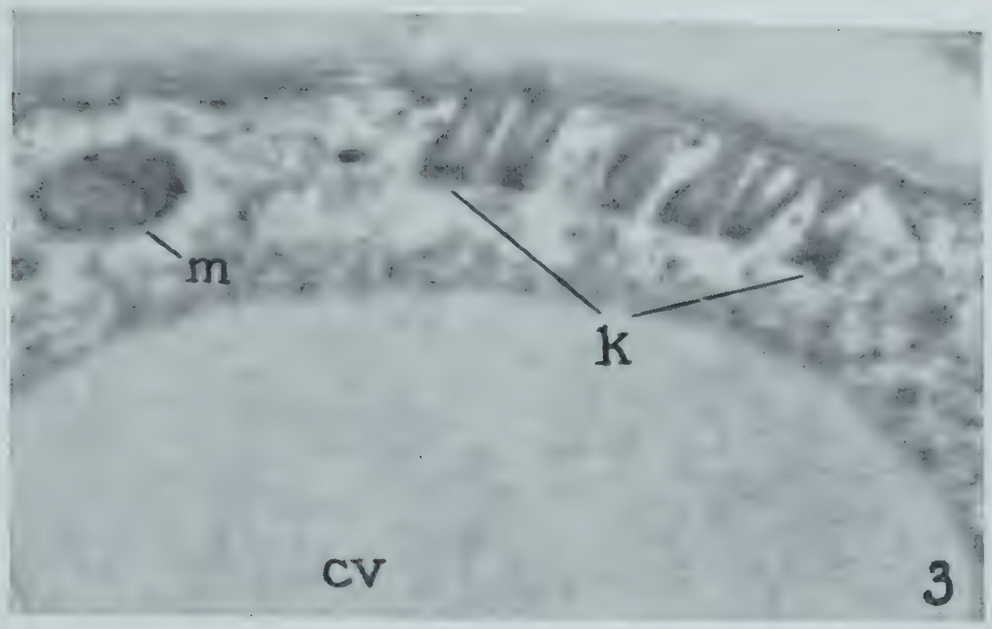


FIG. 3. Electron micrograph of kinetosomes (*k*) (without cilia) in longitudinal section in an adult *Tokophyra*. The kinetosomes appear always in the vicinity of the contractile vacuole (*cv*) of the adult *Tokophyra*. (Compare these aciliate kinetosomes with kinetosomes which are connected with cilia in Fawcett's article: 'Cilia and Flagella.") A mitochondrion is indicated at *m*. Magnification: ×22,400 (M. A. Rudzinska, unpublished.)

granules participating in different functions. However, some of these differentiations are irreversible and a kinetosome which has formed a trichocyst is no longer capable of division (Lwoff, 1950). On the other hand, no present evidence suggests that the dividing kinetosomes distributed over the surface of the cell are differentiated in any fundamental sense; so far as is known, any kinetosome capable of division can replace any other kinetosome. If the kinetosomes are "genetic elements," those within a single cell normally comprise a genetically homogeneous population.

2. *Kineties*

The kinetosomes are not usually distributed at random in the cell cortex, but are organized in progressively more complex hierarchies. The most fundamental unit of organization is the linear array, called the *kinety*, usually extending longitudinally in the cell (Fig. 1). Within the kinety the kinetosomes are distributed at regular distances and are connected by fibers which are located parallel to, and to the right of, the line of kinetosomes (Chatton and Lwoff, 1935; Metz *et al.*, 1953). The number and distribution of kineties at a particular time in the life cycle are characteristic for a given species, but variations may occur with changes in nutritional state or alterations of phases in forms with com-

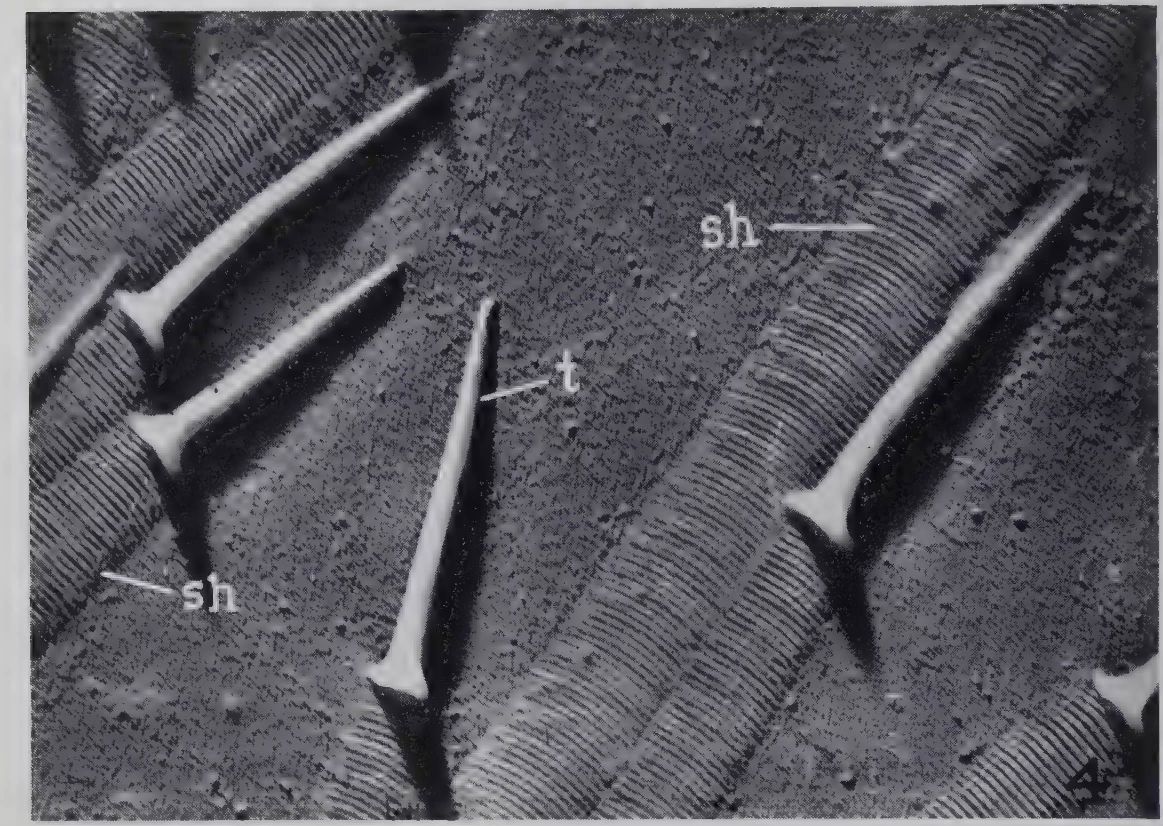


FIG. 4. Electron micrograph of extruded trichocysts from *Paramecium,* shadow-cast with chromium. Each trichocyst is composed of two distinct parts: a sharp, dense tip (*t*) and a long shaft (*sh*) which is regularly striated. Magnification: ×14,104. (Jakus, 1946.)

plex life histories. Thus, in *Ichthyophthirius multifiliis,* a parasitic hymenostome ciliate, the mature feeding stage (trophont) has approximately 2040 kineties, but the hunting stage (theront) has only 43 kineties (Mugard, 1947). Even when the number of kineties remains constant, their distribution may undergo marked alterations; in *Foettingeria actiniarum,* for example, the kineties of the dividing stage (tomont) are simple parallel lines running with the longitudinal axis, but in the feeding stage they are long and tightly coiled about the cell (Lwoff, 1950). Growth of a kinety occurs by intercalary division of kinetosomes yielding an elongation. New kineties are produced directly from old kineties, either by transverse division or by the production of a new line of kinetosomes parallel to the original kinety. Since a kinety seems to serve as a template for the production of a new kinety, it—like the constituent kinetosomes—may be considered a self-reproducing element.

3. *Intrakinetal Differentiation*

The functions of parts of a single kinety vary with their disposition along the linear array, and different derivative structures may be pro-

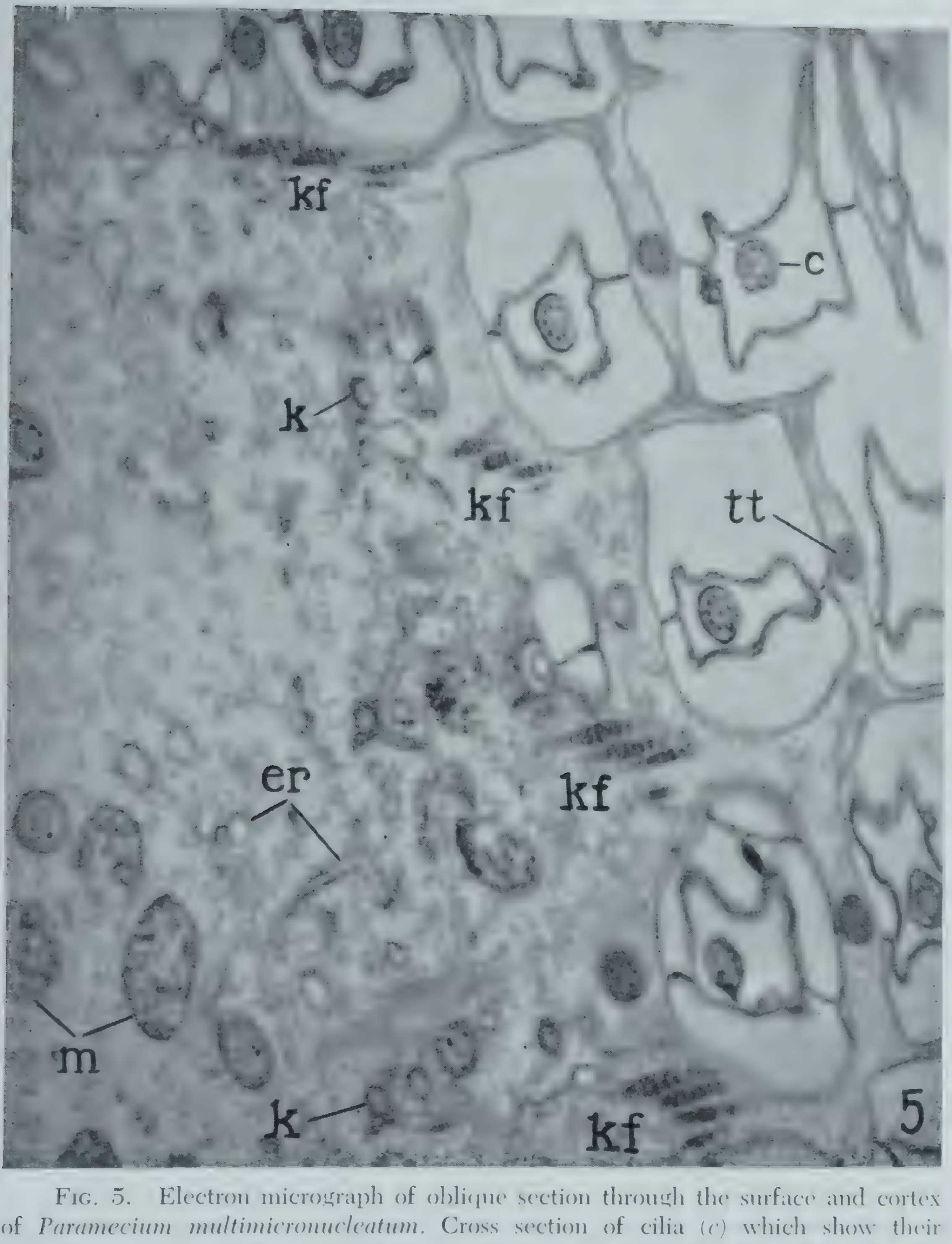


FIG. 5. Electron micrograph of oblique section through the surface and cortex of *Paramecium multimicronucleatum*. Cross section of cilia (*c*) which show their internal structure and arrangement in parallel rows are evident to the right when the plane of section leaves the organism. In line with the cross sections of the cilia and in a deeper plane of the section, cross sections of the kinetosomes (basal bodies) (*k*) are evident. Portions of four groups of kinetodesmal fibrils (*kf*), in longitudinal section, show a regular striation. They run parallel to the kinetosomal and ciliary meridians; *tt*, tip of trichocyst; *m*, mitochondria; *er*, endoplasmic reticulum. Magnification: × 22,500. (Sedar and Porter, 1955.)

duced at different levels. In spite of their different functions the segments can be shown to be equivalent in potentialities. At cell division, when the anterior and posterior sections are separated into different cells, each section yields all the usual structures. The regulation of performance along the kinety may be viewed as a consequence of an anterior-posterior gradient, with the anterior end "dominating" the entire kinety. That this gradient is in fact a kinetal (or localized cortical) gradient rather than a cellular gradient is suggested by experiments employing microsurgery. A kinety reversed from its usual cellular orientation maintains its original polarity in function (Weisz, 1951). The maintenance of the dominance relationships appears to depend on the structural integrity of the cortex; a simple transverse cut in the cortex may be sufficient to destroy the anterior dominance and to establish immediately a new center of dominance just posterior to the cut; healing of the cut results in a suppression of the new center (Weisz, 1951).

4. Interkinetal Differentiation

In most ciliates the kineties within a single cell are differentiated; some are long and some are short; some are characteristically associated with special cytoplasmic organelles such as the cell anus or the contractile vacuoles; some are responsible for the elaboration of the complex oral membranelles. Because of these characteristic associations and functions the kineties may be individualized, named, and located precisely in all the individuals of the species. Since the corresponding kineties of the daughter cells are derived from the same parental kinety and perform the same functions cell generation following cell generation, one might suppose that the individualized kineties are genetically distinct. That this is not so has been demonstrated by extirpation experiments. Kineties removed by microsurgery are replaced after a period of time by kineties which would not ordinarily perform such functions (Weisz, 1951). The special properties of particular kineties do not, therefore, derive from intrinsic differences in their composition, but from some pattern which exists at the level of the cell itself.

A dominance pattern in interkinetal differentiation similar to that in intrakinetal organization can be demonstrated (see Tartar, 1956c). In many ciliates this dominance is associated with a particular kinety, designated as kinety I or the "stomatogenous kinety" (Fig. 1). So long as this kinety persists, all other kineties are suppressed from performing its special functions; when it is removed the next kinety on the left takes its place. The latitudinal organization of the cell may then be conceived as a reflection of a gradient originating at one particular kinety and circumscribing the cell. In *Stentor* this gradient finds morphological expression in the width of pigmented bands between the kineties. The bands immediately to the right of kinety I are very narrow, and successive stripes become progressively broader until the broadest stripes are those just to the left of kinety I. Kinety I, therefore, must occupy a position of discontinuity since the stripes on either side of kinety I are very different in width. The significance of this discontinuity has been demonstrated in experiments of Tartar (1956a, b, c), who has shown that surgically constructed discontinuities—even when involving stripes other than kinety I—call forth the activities normally associated with kinety I. The nature of the right-to-left gradient, and hence the nature of the discontinuity, is not known.

5. *Cytoplasmic Organelles*

Particularly in the higher ciliates, organelles of great complexity may be associated with the kinetosomes. In some instances the organelles are destroyed at cell division and are elaborated anew through the proliferation of kinetosomes from one or more kineties. The kinetosomes in such instances initially form an "anarchic field" which becomes organized and differentiated to produce the particular structure required. In other cases the organelles persist through successive cell divisions and the extra organelles required for the additional cells are produced from anarchic fields of kinetosomes arising as buds from the original structure. These specialized structures are, therefore, like kinetosomes and like kineties, self-reproducing structures. Again, however, one may not conclude that the kinetosomes in these structures are genetically distinct from those in other parts of the cell. One primary exception to this conclusion appeared to be the failure of replacement of the oral organelles in *Paramecium* when they were removed. Tartar (1954), however, has shown that starved paramecia are unable to regenerate parts, even parts of relatively slight complexity and parts easily regenerated when the cell is fed, and a paramecium deprived of an oral apparatus is *ipso facto* a starving paramecium. Moreover, Hanson (1955) may have obtained the replacement of oral structures in *Paramecium* from extraoral materials. He constructed double animals with two sets of oral apparatus; he then destroyed one set of organelles with an ultraviolet microbeam, leaving the other to provide nourishment to the doublet. Under these conditions the destroyed mouth parts were regenerated, possibly from the materials in the vicinity of the original mouth.

We are thus led to the conclusion that the kinetosomes, however different in function and regardless of the patterns in which they are organized, are essentially equipotent (Sonneborn, 1950a). A primary characteristic of genetic material, namely the ability to change (mutate)

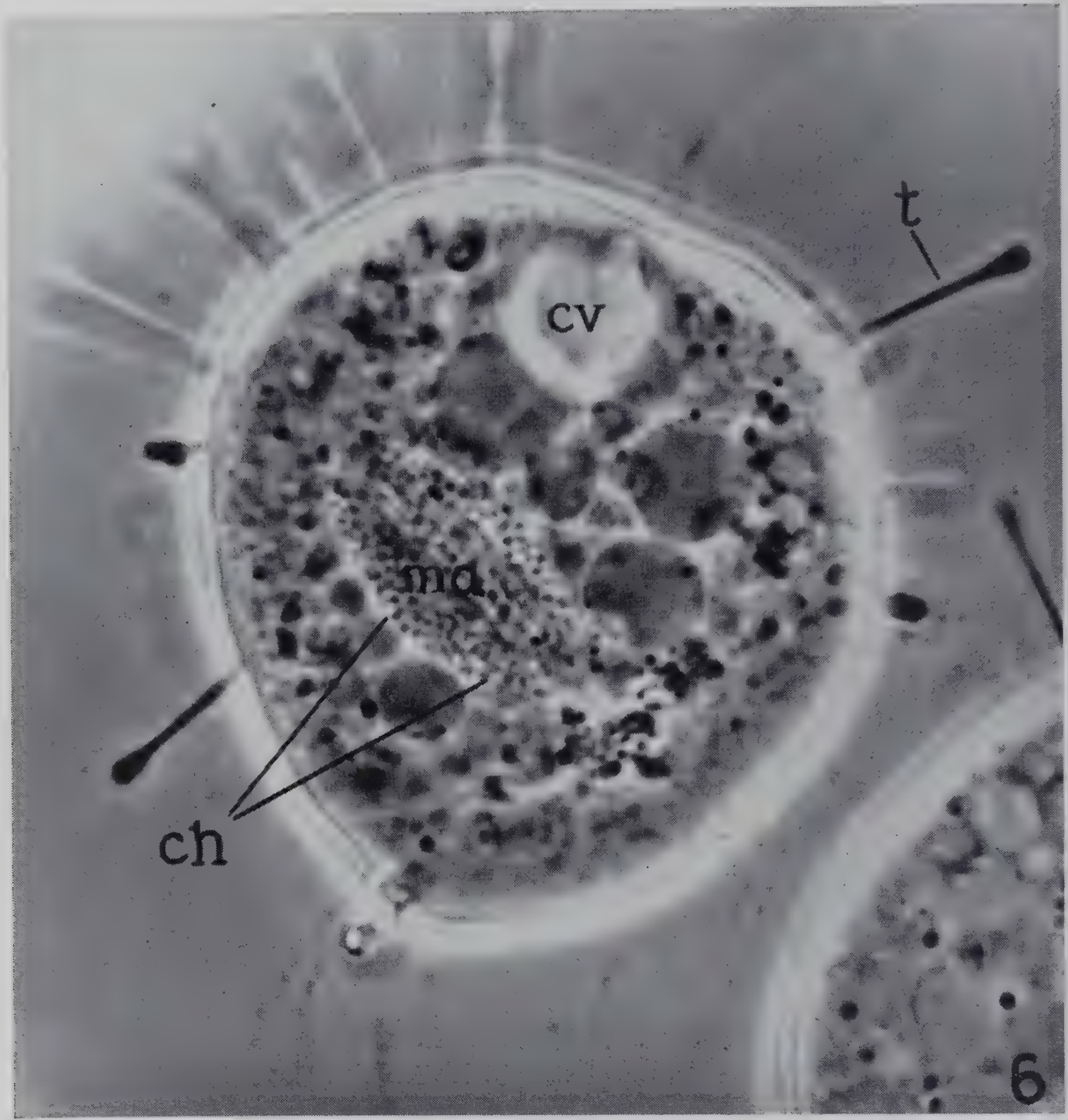


FIG. 6. Photomicrograph of phase contrast image of living *Tokophyra infusionum*. The macronucleus (*ma*) is centrally located and oblong in shape. It contains a large number of chromatin bodies (*ch*) which appear as refractile granules; *t*, tentacle; *cv*, contractile vacuole. Magnification: × 1,760. (Rudzinska and Porter, 1955.)

and to reproduce itself in the altered form, has not been demonstrated for these elements. Most significantly, Preer (1959) has recently found that an altered type of trichocyst in *Paramecium* is due to a gene mutation; this suggests that the specific properties of kinetosomal derivatives have their bases in nuclear rather than in cytoplasmic elements. The various cortical patterns are also maintained and reproduced faithfully, but must be denied true genetic continuity since they can arise in the absence of a pre-existing pattern of the same sort; the prior subpattern

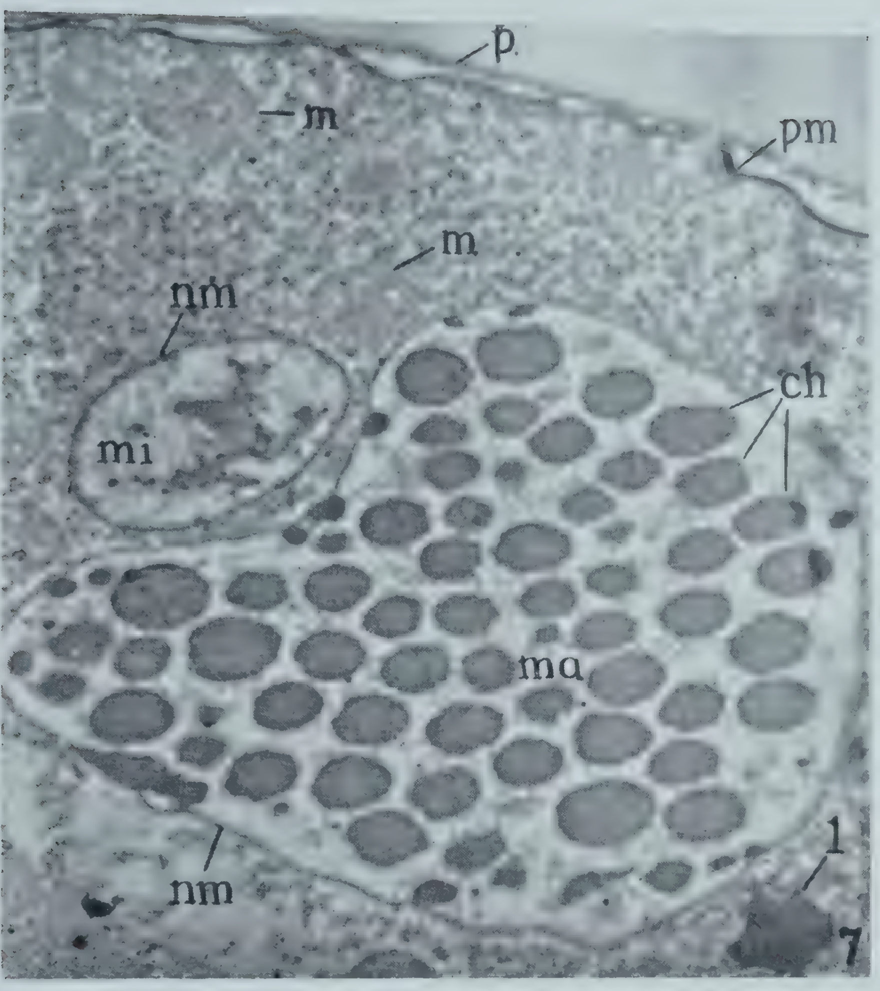


FIG. 7. Electron micrograph of section through the macro- and micronucleus of *Tokophyra infusionum*. Both nuclei are surrounded by a double nuclear membrane (*nm*) which may be seen at higher magnifications in places in which the section is perpendicular to the membranes. In the macronucleus (*ma*) dense oval profiles represent chromatin bodies (*ch*). They are embedded in a matrix of low density. The oval micronucleus (*mi*) lies in an indentation of the macronucleus. The dense chromatin material in the micronucleus is irregular in shape. The matrix shows a low density; *p*, pellicle; *pm*, plasma membrane; *m*, mitochondria; *l*, lipid body. Magnification: ×5,810. (M. A. Rudzinska, unpublished.)

may be useful, but it is not essential. The distribution and functional diversification of the kinetosomes reflect the cellular organization, but their role in determining that organization is not well established. That cellular patterns are influenced markedly by the nuclear apparatus will be discussed in the following sections.

B. The Nuclear Apparatus

Most ciliates contain two kinds of nuclei (Fig. 7)—one or more small nuclei (micronuclei), and one or more large nuclei (macronuclei). Micro-

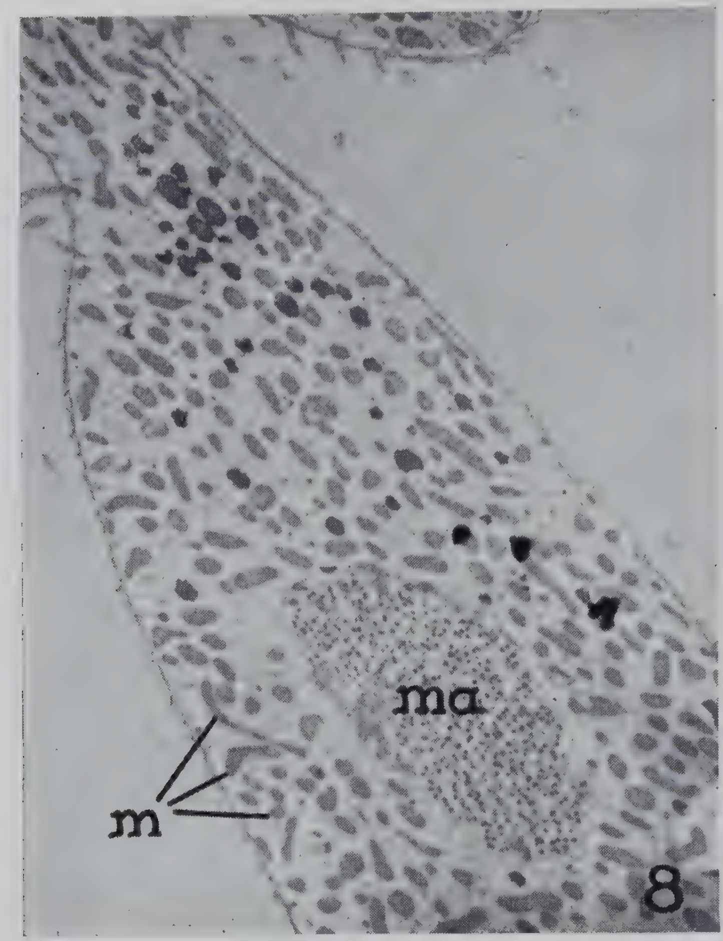


FIG. 8. Low-power electron micrograph of *Tetrahymena pyriformis* (amicronucleate strain). The macronucleus (*ma*) appears as an oval body surrounded by a double nuclear membrane. The dense small granules represent the chromatin bodies; the larger, the nucleoli. In the cytoplasm numerous mitochondria (*m*) may be seen. Magnification: ×3500. (Sedar and Rudzinska, 1956.)

nuclei (Figs. 7, 10) and macronuclei (Figs. 6–10, 24, 25) differ not only in size, but in biological activity. The micronuclei are usually considered the reserve or germinal nuclei and periodically give rise to macronuclei through the processes of nuclear reorganization. The macronuclei are the nuclei primarily concerned with the continuing metabolic activities of the cell and are discarded at nuclear reorganization; macronuclei in most ciliates appear incapable of giving rise to micronuclei. The evidence for the primacy of macronuclear functions in vegetative growth is of several sorts. Naturally occurring amicronucleate strains (Figs. 8, 9) have been observed in a number of ciliates, and these may make up a large fraction of wild cultures in some species. The loss of micronuclei from normally micronucleate strains has been observed or induced in the laboratory, and in certain instances this loss appears not to impair seriously the ability for continued growth or for normal morphogenesis.

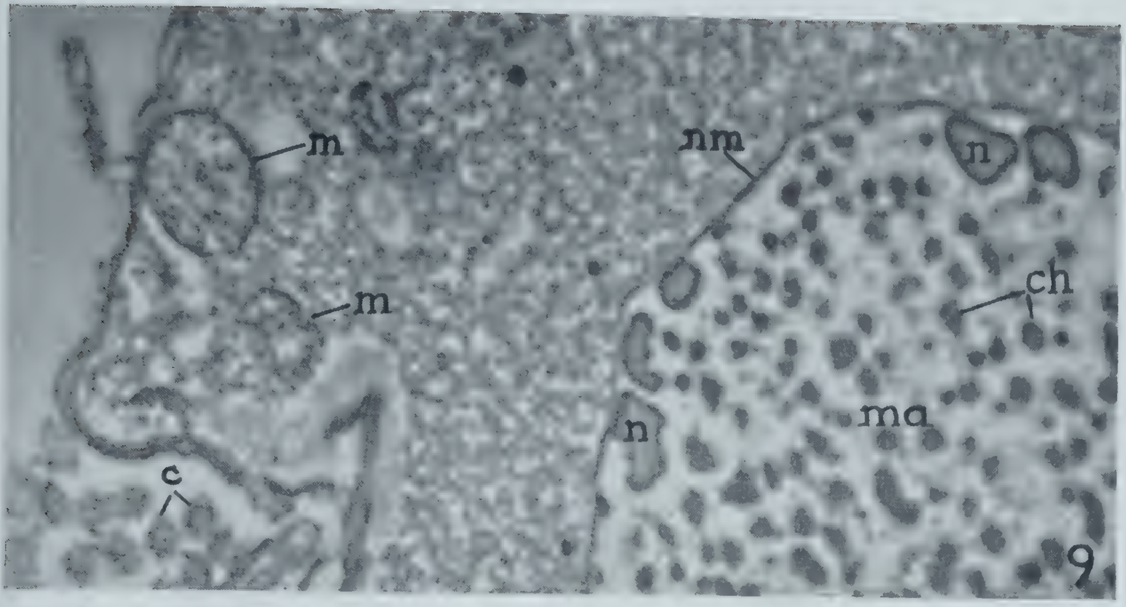
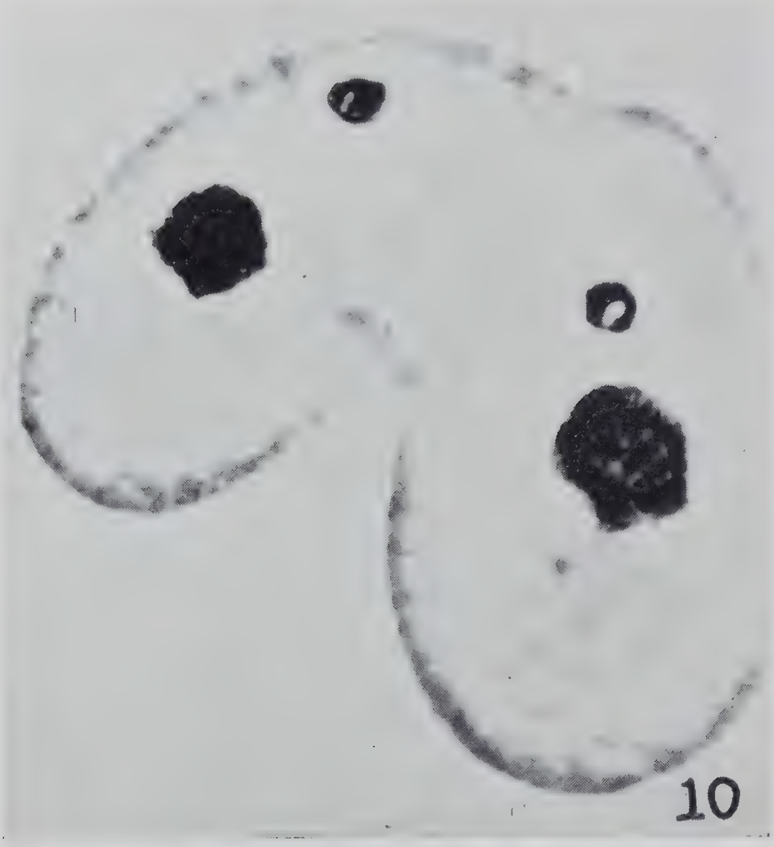


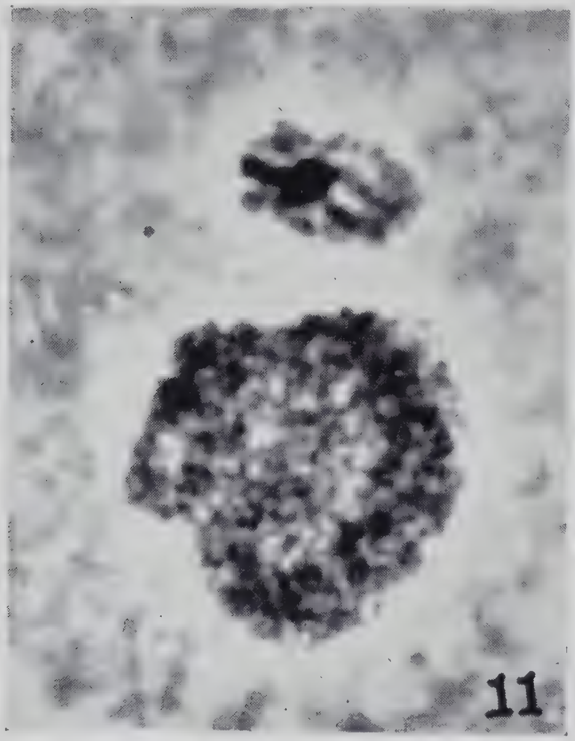
FIG. 9. Higher-power electron micrographs of part of macronucleus of amicronucleate strain of *Tetrahymena pyriformis*. Note the difference in size between the chromatin bodies (*ch*) and nucleoli (*n*). In addition, the nucleoli appear to be denser on their periphery; the chromatin bodies show a homogeneous density. The nuclear matrix is of low density. Mitochondria (*m*) show a fine structure similar to that of mitochondria in metazoan cells. They are surrounded by an outer limiting membrane and an inner membrane which by its extensions forms the internal mitochondrial structures. While in the majority of cells in higher organisms the internal structure consists of ridges, cristae mitochondriales, in *Tetrahymena* as in other Protozoa they appear prevalently as fingerlike projections or microvilli. This is indicated by the presence of oval and circular profiles. The nuclear membrane is indicated at *nm*; cross sections through cilia at *c*. Magnification: ×9,660. (M. A. Rudzinska, unpublished.)

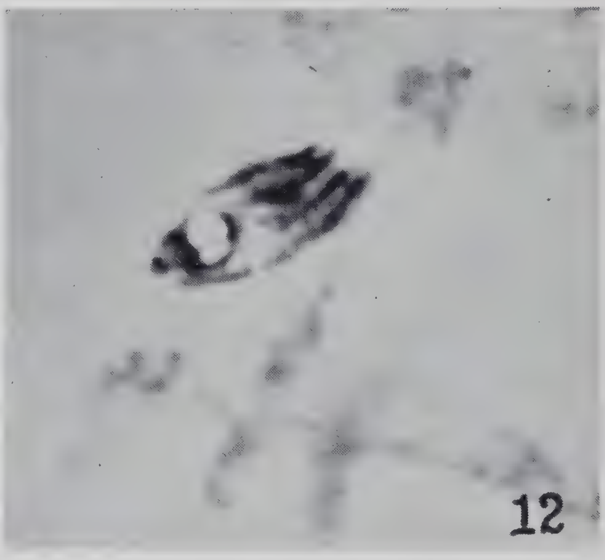
In other cases growth and morphogenetic activity are curtailed with the removal of the micronucleus; in these instances the micronuclei clearly have a vegetative function distinct from that of the macronucleus, but this function cannot be general in view of the many exceptions. In contrast to the equivocal status of the micronucleus in vegetative functions, no instance of growth or morphogenesis is known in the absence of macronuclei (Balamuth, 1940; Weisz, 1954). Experimentally induced "heterokaryons" with different genetic compositions for the micronuclei and the macronuclei express the macronuclear genotype and no traces of specific micronuclear effects have been demonstrated, even when sensitive tests for such effects have been devised (Sonneborn, 1947; unpublished). This relative inertness of the micronuclei may not, however, reflect any fundamental differences in the activity of the two kinds of nuclei, but only the relative quantities of nuclear material. Both kinds of nuclei have idiosyncrasies, and neither is very well understood. The



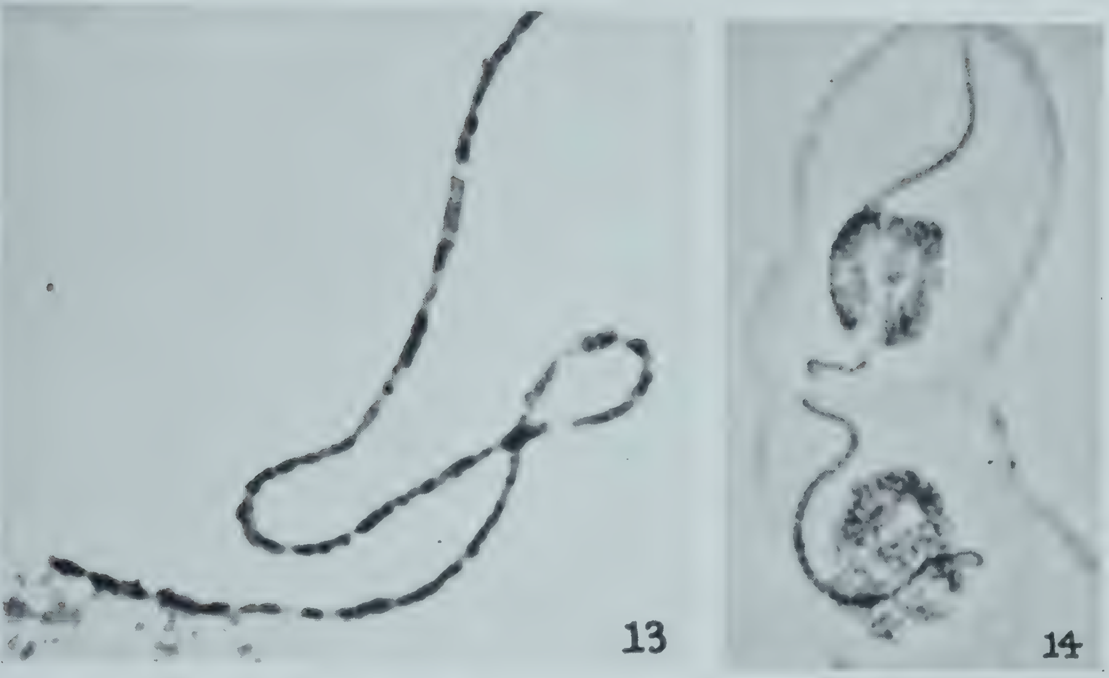
Figs. 10–19 represent photomicrographs of the chromosome behavior of *Tetrahymena pyriformis* (strain WH-6, and WH-14, mating types I and II, respectively, of variety 1). (Ray, 1956.)

Fig. 10. Beginning of conjugation. Magnification: × 1400.





Figs. 11, 12. Appearance of the "parachute" shape of the micronucleus. Magnification: × 3200.



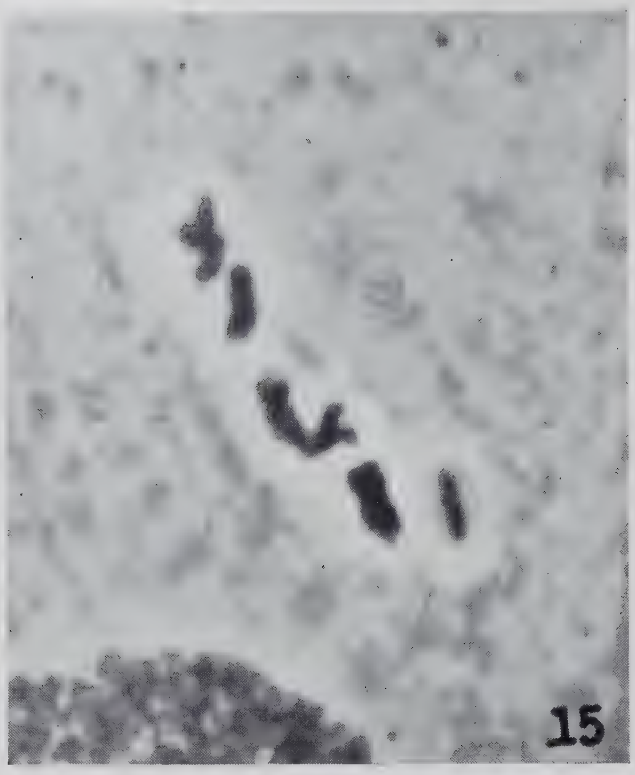
FIGS. 13, 14. Crescent stage of micronucleus. Magnification: ×2,700 and 930 respectively.

chief problem, which will be discussed below, is the reconciliation of conventional genetic behavior with apparently atypical cytological behavior.

1. The Micronuclei

The micronuclei (Fig. 7) are small nuclei, usually not larger than a few microns in diameter. Their number varies with the species and may range from one to several dozen. Perhaps because of their small size, satisfactory studies of their behavior in mitotic divisions are rare and the studies which have been undertaken yield observations difficult to interpret. Seldom have the cytological studies produced clear evidence for discrete individualized chromosomes, although mitotic spindles are formed and the chromatin is divided equally to the daughter nuclei. In spite of the obscurity surrounding the micronuclear divisions, the genetic consequences of the process are unequivocal; genetic material is reliably and equally transmitted to daughter nuclei.

The chief time in the life cycle when individualized chromosomes are clearly seen is the time of nuclear reorganization when the micronuclei undergo meiosis. Even here, however, the processes are atypical, and the first meiotic division usually includes an extended stage in which the nuclei take the form of "parachutes" (Figs. 11, 12) or "crescents" (Figs. 13, 14). One of the clearest accounts of chromatin behavior during



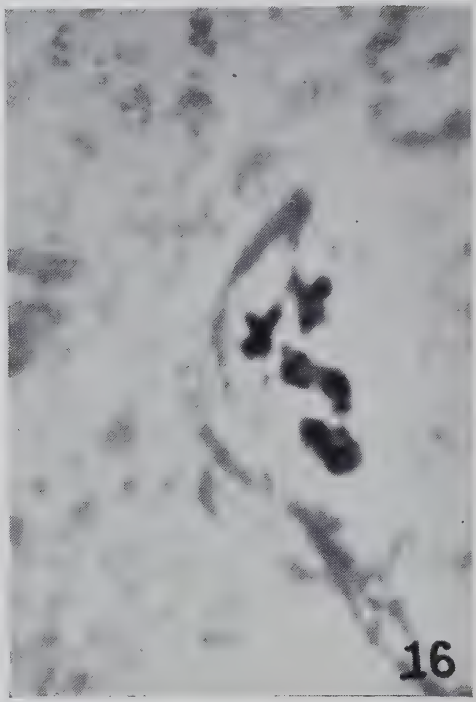
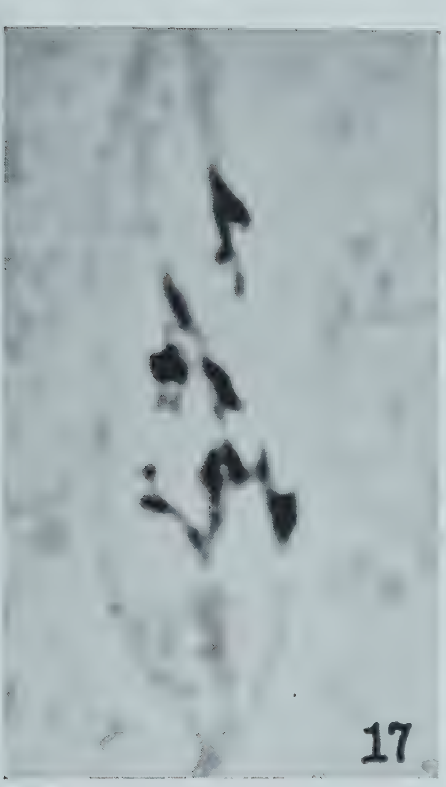


FIG. 15. Five bivalent chromosomes arranged in linear order along the elongated micronucleus. Magnification: × 3200.

FIG. 16. Prometaphase of first meiotic division. Magnification: × 3200.

meiosis is found in the recent description of Ray (1956) based on the unusually favorable material provided in *Tetrahymena pyriformis* (Figs. 10–19). At the initiation of meiosis the chromatin appears first as tangled threads, initially single but subsequently paired (Figs. 11, 12). The nucleus then begins to elongate into a narrow cylinder, eventually longer than the cell itself and coiled around the macronucleus: this is the crescent stage (Figs. 13, 14). Within the crescent the chromatin appears as a thick doubled thread which may be a continuous structure or which may be made up of chromosomes lined up end to end. The crescent then begins to shorten, and as it shortens discrete chromosomes appear in the form of five conventional tetrads, with characteristic lengths and spindle attachment points (Figs. 15, 16). These tetrads may not form a clearly defined metaphase plate, but behave as semiautonomous elements, each dividing and sending a dyad to each pole without a preliminary orientation and only in approximate synchrony (Fig. 17). The second meiotic division (Figs. 18, 19) and a third prezygotic mitotic division appear to be typical of meiotic and mitotic divisions in most other organisms. Following fertilization and the establishment of the diploid condition, the expected ten chromosomes have been observed at least during the first postzygotic nuclear division. Following the differentiation



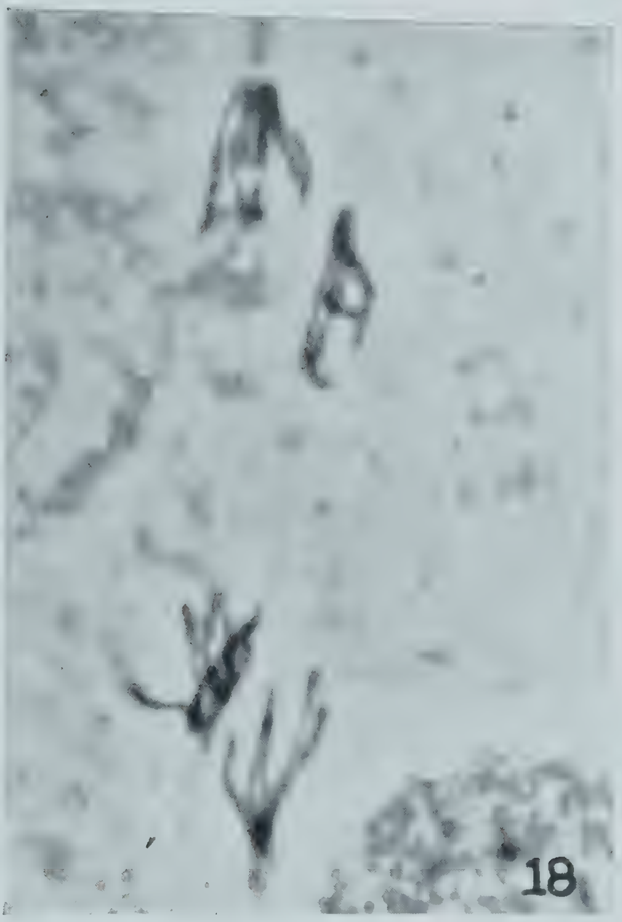


FIG. 17. First anaphase—dyads may be recognized. Magnification: ×3200.
FIG. 18. Second anaphase. Magnification: ×3200.

of nuclei into definitive micronuclei and macronuclei, however, discrete chromosomes disappear from view and can be clearly seen again only when meiosis is again induced.

This account of meiotic events is similar to that reported for other ciliates but is more conventional in certain respects. Devidé and Geitler (1947) summarize the studies on a group of organisms including *Chilodonella, Colpidium, Euplotes, Stylonychia,* and *Vorticella,* which show a progressive reduction in the number of chromatic elements following the first meiotic division. They suggest that the chromosomes in the micronuclei of ciliates are frequently aggregated into "superchromosomes" perhaps made up of end-to-end associations of the discrete chromosomes visible during and immediately following meiosis. Perhaps the discrepancy between these reports and that on *Tetrahymena* is due to a difference in the time at which the reaggregation occurs. If the chromosomes of vegetative micronuclei are regularly aggregated into one or a few extended chromatinic bodies, the difficulty in seeing discrete chromosomes at mitosis is more understandable. Moreover, the strange nuclear devices

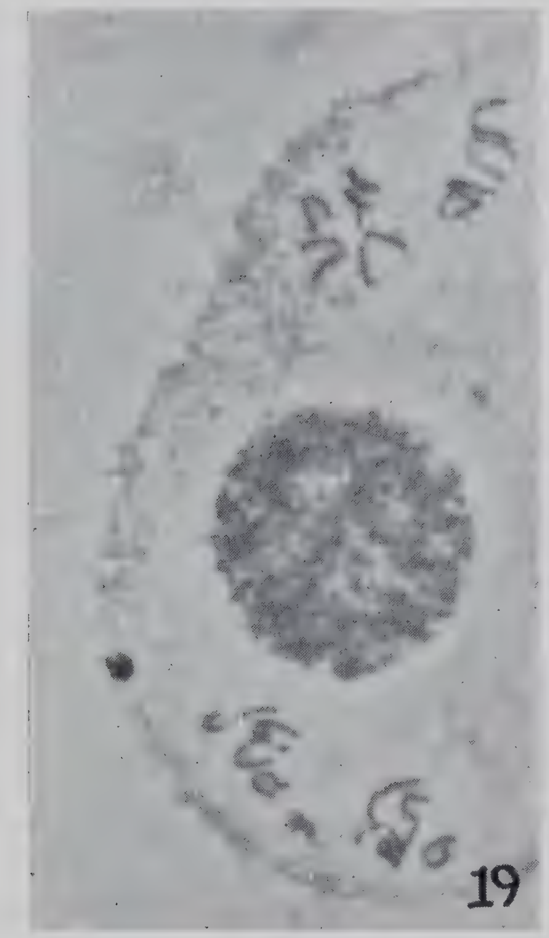


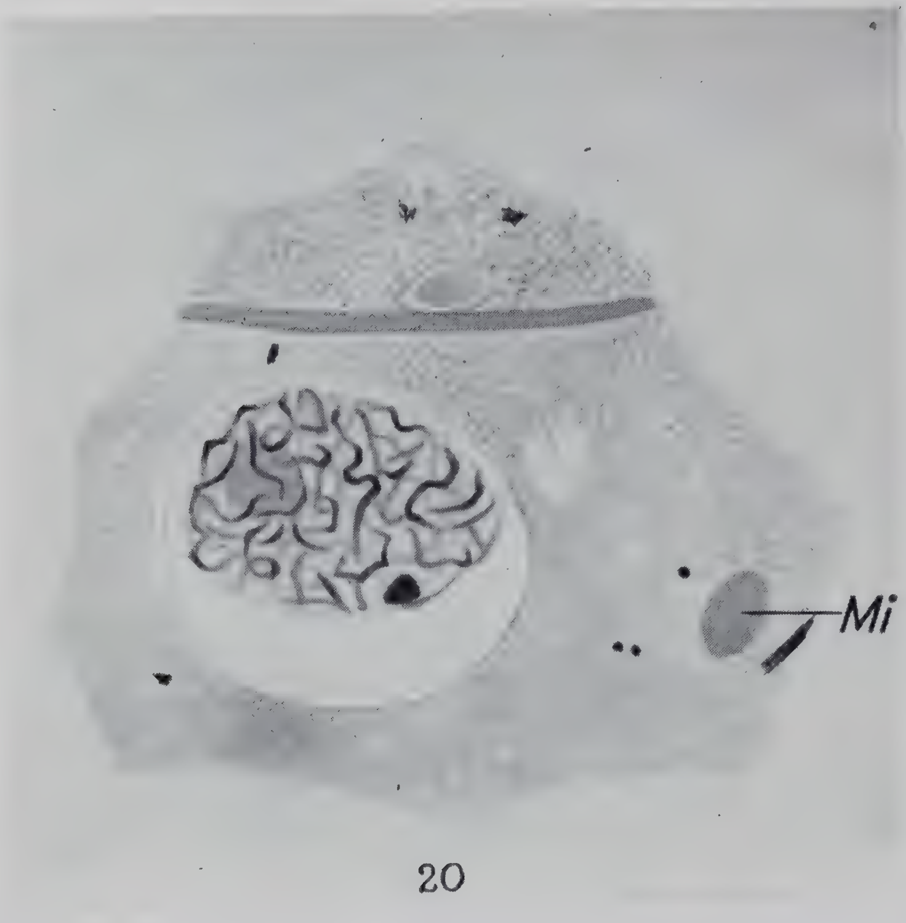
FIG. 19. Haploid complement of five chromosomes at each of the four poles at second anaphase. Magnification: × 1400.

observed in the first meiotic division may be explained as mechanisms for achieving the re-establishment of individual chromosomes in preparation for genetic recombination. In any case, the general occurrence of chromosome aggregates in the micronuclei may offer an important clue to the organization of the chromatin in the macronuclei.

2. *The Macronuclei*

The macronuclei (Figs. 6–9, 25) may occupy fifty or more times the volume of the micronuclei and may contain a correspondingly large amount of deoxyribonucleic acid (DNA) (Moses, 1950). In some organisms the macronucleus is a single spherical body; in others it may form an elongated cylinder or a series of nodes extending the length of the cell and connected by thin strands of nuclear material. Other forms have multiple separate nuclei, the number ranging from two to several hundred depending on the species. The large size and the large amounts of DNA clearly demonstrate that the macronucleus is a "multiple" nucleus in some sense. However, it divides by what is apparently a simple constriction process and usually provides no clear evidence for mitotic spindles (but see Schwartz, 1957), condensed chromosomes, or other paraphernalia of a normal mitosis. By this, one should not infer that no evidence for intranuclear reorganization is available, for many workers have reported "reorganization bands," cycles of nucleolar production, and other incompletely understood changes. In spite of the confusion, and in spite of the lack of morphological evidence for regular chromosome behavior, genetic evidence for precision in the transmission of genetic information is good. Even fragments of macronuclei produced by macronuclear breakdown at conjugation (Sonneborn, 1947), or produced during budding (Grell, 1950), or isolated during microsurgical operations (Weisz, 1954) are capable of regenerating whole normal macronuclei. Since this precision is apparently not based on a standard form of mitosis, other possibilities have to be considered.

One interpretation (Kimball, 1943) suggests that the macronucleus is polyploid, but differs from ordinary polyploid nuclei in the lack of coordination of chromosomal division and distribution. According to this view the division of the macronucleus consists of a random assortment of the individual chromosomes into approximately equal samples. The adequacy of this explanation depends in part upon the total ploidy of the macronucleus. If the number of sets of chromosomes is high, the precision of transmission of genetic traits should approach that afforded by a mitotic mechanism; if the number of chromosome sets is low, inequalities among daughter nuclei should be common and genetic segregation during macronuclear division should be demonstrable. A test for the interpretation then depends upon the development of a stochastic model relating segregation to ploidy levels under conditions of random assortment, an experimental determination of the amount of genetic segregation which occurs, and an independent estimate of the ploidy level of the macronucleus. These requirements for an adequate test have been partially met in studies on *Paramecium aurelia*. In extensive studies both on normal growth and on fragment regeneration (which provides an especially critical test for the interpretation), Sonneborn (Sonneborn *et al.*, 1956; unpublished) has failed to find any cases of genetic segregation in the macronucleus. Making the most conservative assumptions, he concludes that the data available are adequate to exclude any hypothesis of random segregation which assumes a ploidy level less than 320. Unfortunately, the relative quantities of DNA in the macronucleus and the diploid micronucleus of *P. aurelia* are not yet known. In *P. caudatum* (Moses, 1950) the ratio is only about 40:1, suggesting a macronuclear ploidy of about 80, but the micronuclei in *P. caudatum* are much larger



FIGS. 20–23 represent stages in the development of the macronuclear anlage in *Ephelota gemmipara.* (Grell, 1953.)

FIG. 20. Development of the macronuclear anlage. Spireme stage. Magnification: ×1950.

than those in *P. aurelia* and the ploidy level in *P. aurelia* may be considerably larger. Nevertheless, the existence of ciliates with macronuclear ploidy levels of 100 or less is clearly established and some of these ciliates have been long maintained without any evidence of genetic segregation in the macronucleus. In particular, strains of *Tetrahymena* heterozygous for mating-type alleles have been maintained for hundreds of cell generations without the change of mating type which would be expected if chromosomal segregation occurred in the macronucleus (Nanney, unpublished). Hence, though not entirely satisfactory, the available evidence supports the notion that some special mechanism exists for maintaining balanced chromosome sets in the macronucleus.

Sonneborn (1947) visualized this mechanism as a subnuclear structure, each subnucleus containing two sets of chromosomes and dividing by a conventional mitotic division whose details are obscured by the lack of synchrony and the large mass of material. However, no morphological evidence has been obtained for a localization of chromosome sets or for

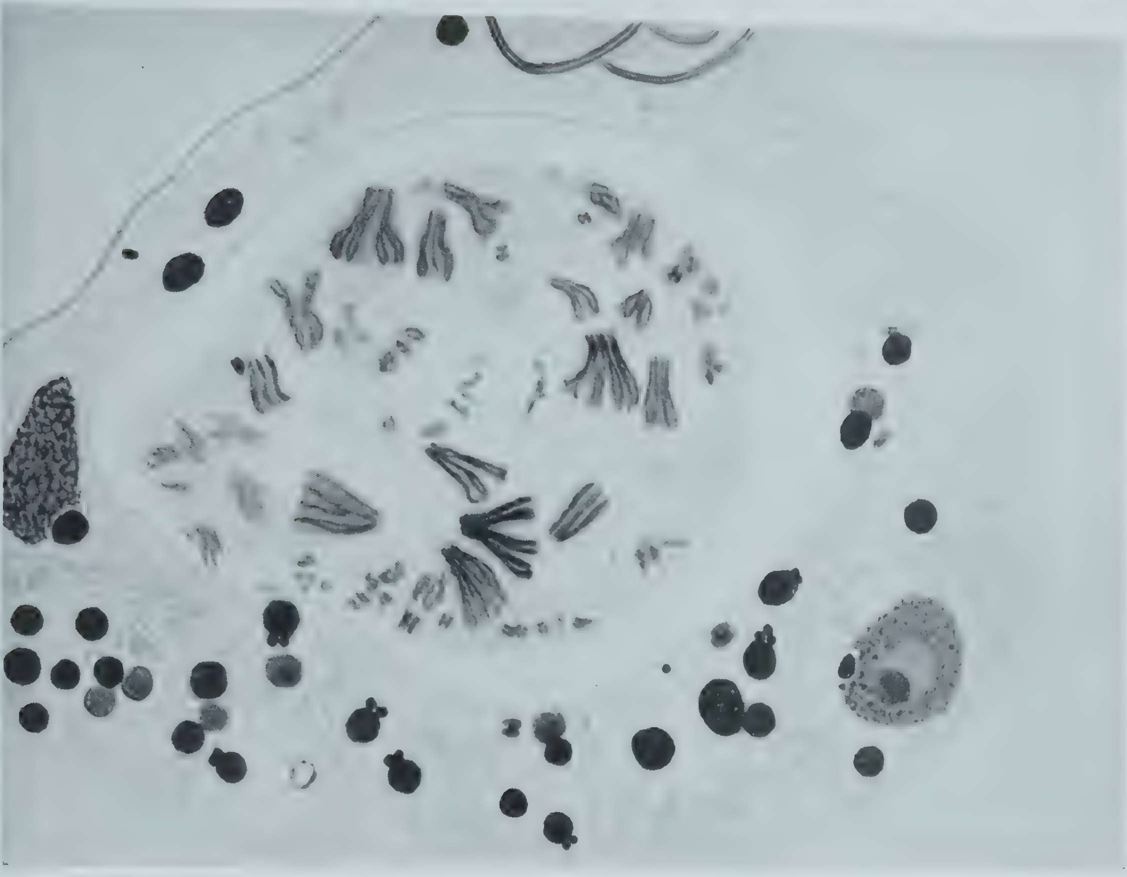
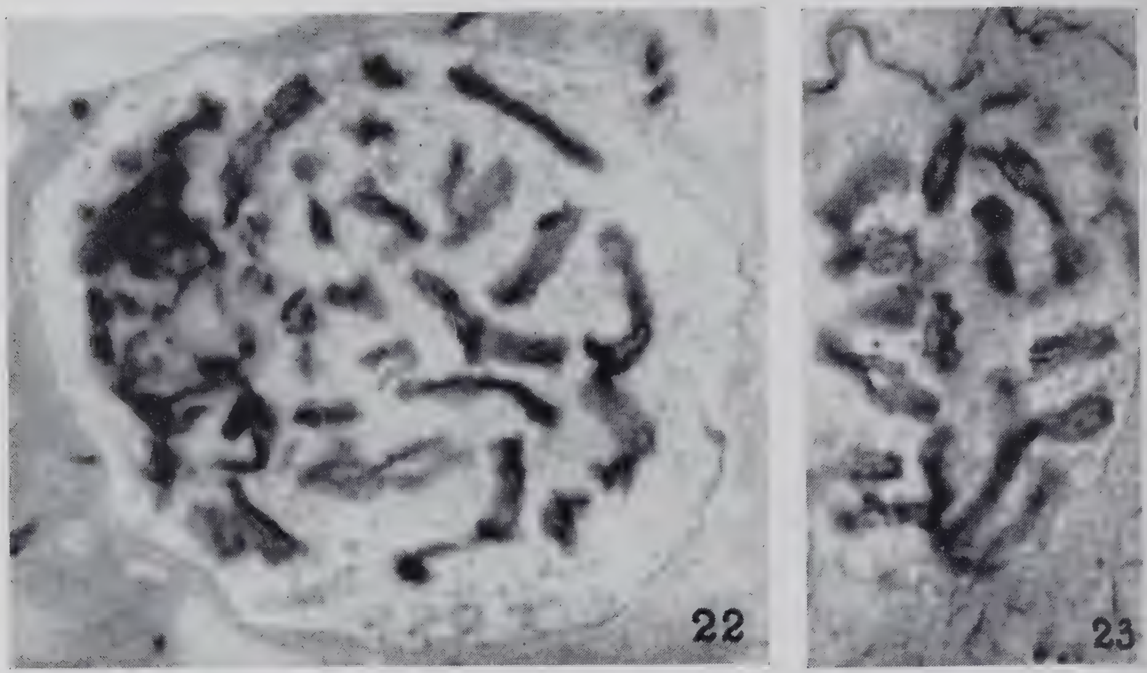


FIG. 21. Endomitotic polyploidization (bundles of chromatic elements).

a partitioning of the macronucleus by intranuclear membranes, even when these were diligently sought. Quite possibly the maintenance of intact diploid genomes is achieved by some means other than compartmentalization into discrete subnuclei. This notion finds some support in the unconventional behavior of the chromosomes in the micronuclei, mentioned above, and also in the few reports of chromosome behavior in the developing macronucleus. Usually, discrete chromosomes are not observed in the macronucleus at any time, but Grell (1949) reports that bundles of chromosome-like elements can be seen in the developing macronuclei of *Ephelota gemmipara* (Figs. 20–23) and that these bundles become progressively more compound as the macronuclei mature. If these are individual chromosomes, they do not appear to be associated into "diploid sets" but into bundles of identical elements. On the other hand, their relatively large and uniform sizes and their small numbers suggest that each element may in fact be an aggregate of chromosomes; if they are aggregates, particularly linear aggregates composed of diploid sets of chromosomes, they may be the morphological equivalents of the "subnuclei" proposed on the basis of genetic evidence. If the chromosomes were physically united in some way, compartmentalization and mitotic



FIGS. 22, 23. Photomicrographs of macronuclear anlage, showing stages of endomitotic polyploidization. Magnification: × 1550.

spindles would not be required to maintain balanced chromosome sets indefinitely in vegetative growth. This interpretation lacks a firm observational basis but appears not unreasonable in view of the evidence for chromosome aggregates in the micronuclei.

3. *Nuclear Behavior*

Throughout the life cycle of ciliates the nuclear and the cytoplasmic cell components undergo complex and integrated modifications. The precision of these correlated alterations suggests constant interaction between the nuclei and the cytoplasm. Some of the cytoplasmic alterations have been discussed earlier and the significant role of the macronucleus in morphogenesis has been mentioned. Cytoplasmic reorganization does not occur in the absence of a macronucleus. Moreover, nuclear mutations affecting morphogenesis and cell form are known. The transfer of material from the nucleus to the cytoplasm has been described in many forms (Ehret and Powers, 1955; MacLennan, 1941), and this material probably serves an important function. Hence the participation of the nucleus in cytoplasmic affairs is not in question, but only the degree to which the cytoplasmic patterns are dependent upon the nucleus and to what extent they are autonomous. We shall return to this problem later.

Nucleocytoplasmic relations cannot, however, be viewed simply as a problem in nuclear control of the cytoplasm. Evidence for cytoplasmic control of nuclear activity has existed since the early observations of

Maupas (1889) on nuclear reorganization in a variety of ciliates. Much of the evidence is circumstantial in that it is based on correlations between the position of a nucleus in the cytoplasm and its behavior, but in recent years the evidence has been extended by experimental manipulations (Sonneborn, 1951, 1954a; Nanney, 1953; Weisz, 1951). To illustrate the general character of this evidence, the nuclear events occurring at conjugation in *Tetrahymena* (Maupas, 1889; Nanney, 1953; Elliott and Hayes, 1953; Ray, 1956) will be briefly described.

Conjugation is initiated by the attachment of cells, usually of diverse mating types, at their anterior ends near the mouth (Fig. 10). The single micronucleus then undergoes meiosis (Figs. 11–19), the details of which were described above, and produces four haploid nuclei. These nuclei migrate to the anterior end, where one becomes attached in a specific location near the mouth. The remaining nuclei (relic nuclei) subsequently move toward the posterior end of the cell, where they disintegrate. This difference in the behavior of identical haploid nuclei is a first example of a correlation between nuclear location and nuclear behavior; the nucleus attached near the oral membranes persists whereas the unattached nuclei drift away, undergo no further divisions, and disappear. The significance of the attachment is emphasized by Sonneborn's (1954a) observations on a mutant stock of *P. aurelia* which is characterized in part by the belated nuclear migration to the oral region; in this stock none of the nuclei makes the attachment and none persists.

Following its attachment, the persistent haploid nucleus undergoes a mitotic division with a spindle oriented at right angles to the cell surface. At the end of this division one of the daughter nuclei remains attached to the surface and becomes the migratory nucleus; the other (the stationary nucleus) is left free in the cytoplasm and comes to rest just anterior to the macronucleus. These two identical nuclei have different functions in the subsequent reorganization. The migratory nucleus moves through the surface into the mate and there fertilizes the stationary nucleus, while the stationary nucleus remains behind to receive the migratory nucleus from the mate. In some ciliates, though not in *Tetrahymena,* the differences in functions are associated with marked morphological alterations; the migratory nucleus becomes extended into a form reminiscent of sperm cells in other organisms and the stationary nucleus rounds up (Dogiel, 1925). Both the functional and the morphological characteristics of the nuclei in these cases are correlated with their location in the cytoplasm.

Following the transfer of the migratory nucleus and its fusion with the stationary nucleus, the synkaryon undergoes its first postzygotic division just anterior to the macronucleus. This division is characterized by an oblique or transverse spindle orientation which leaves both daughter nuclei in the anterior half of the cell. These nuclei then move posteriorly and undergo a second division behind or to the side of the macronucleus.

The spindles formed at this division are oriented longitudinally in the cell and extend eventually from one end of the cell to the other. This difference in spindle orientation in the two postzygotic nuclear divisions may be attributed to differences in the cytoplasmic environments anterior and posterior to the macronucleus. A first postzygotic spindle has the longitudinal orientation when it is displaced posteriorly by centrifugation (Nanney, 1953).

The longitudinal spindle leaves daughter nuclei at opposite ends of the cell and these daughter nuclei have different prospective fates. Those at the anterior end begin to enlarge into macronuclei while those at the posterior end become the micronuclei. The significance of the cytoplasmic positions of the nuclei is shown by the alterations of their presumptive fates by centrifugal reorientation (Nanney, 1953). Anterior nuclei moved to the posterior end become micronuclei and posterior nuclei moved to the anterior end become macronuclei. In *P. aurelia* the situation is similar in principle (Sonneborn, 1954a). Here the anterior nuclei normally become micronuclei and the posterior nuclei become macronuclei; again centrifugation can alter both the number of postzygotic nuclear divisions and the course of differentiation of their products.

These observations leave little doubt that the cytoplasm plays an important role in directing nuclear activities. They leave unanswered, however, the problem of the nature of the cytoplasmic influences and, more importantly, the control of the cytoplasmic patterns. Even during their complex alterations the nuclei are acting upon the cytoplasm and bringing about specific alterations in it. This is suggested by a number of observations in *P. aurelia*. Meiosis and the subsequent micronuclear alterations do not occur at all in the absence of the macronucleus (Sonneborn, 1955); if the cytoplasmic patterns were entirely autonomous this would not be expected. Secondly, the fate of the old macronucleus is apparently determined by the activities of the new macronuclei. In *P. aurelia* the macronucleus breaks down into fragments; in the presence of normal new macronuclei these do not grow or divide, but are diluted out in the fissions following nuclear reorganization. When, however, the new macronuclei are aborted or retarded in their development, the old fragments begin to grow and can reconstitute a whole macronucleus (Sonneborn, 1947). The suppression of growth in the old macronucleus is effected by the developing new macronucleus, undoubtedly operating through the cytoplasm. These observations clearly demonstrate the existence of a sequence of nucleocytoplasmic interactions in which progressive interdeterminations of the cytoplasm by the nucleus and of the nucleus by the cytoplasm can be inferred. Undoubtedly the macronucleus plays a significant role in directing the entire sequence, including in the final analysis its own destruction.

III. CELLULAR VARIATIONS AND THEIR BASES

One of the chief contributions of studies with the ciliates to general problems of cellular biology has been the analysis of persistent cellular variations. The most extensive experiments have been conducted with *Paramecium aurelia* and its near relatives, and the following discussion is largely restricted to these forms. The success of such studies has been to a large extent determined by the special features of ciliate biology discussed above. Intraclonal variations of several different kinds have been observed, and—unlike such variations in the cells of higher organisms—these may be studied by breeding analysis. Moreover, the special features of conjugation permit an elegance of discrimination between different kinds of physical bases which is not matched in other forms in which differentiated cells may be crossed. Specifically, conjugation is a process in which karyogamy occurs, but in which cytoplasmic admixture often does not occur. Under special circumstances, however, a mixture of cytoplasm can be experimentally induced, and the influence of the cytoplasm in persistent variations may be evaluated.

A. Variations Dependent on Particulate Cytoplasmic Elements

The most familiar and best-understood example of intraclonal variation in the ciliates is probably that associated with the "killer" traits in *P. aurelia* (Sonneborn, 1947, 1950b, 1959; Beale, 1954). Some strains (killers) in this species produce a toxin (paramecin) which is capable of killing cells of other strains (sensitives). This toxin is released from the killer cell as discrete units, a single one of which may be sufficient to kill a sensitive cell (Austin, 1948). The toxin does not multiply within the sensitive cell, nor can it be recovered from killed sensitive cells.

The ability to produce the toxin is ordinarily transmitted with great reliability during vegetative growth, but under certain circumstances killer clones can produce sensitive cells which breed true subsequently. Some of the conditions capable of inducing the transformation of killers into sensitives are rapid growth (Preer, 1948), treatment with certain antibiotics (Brown, 1950), and treatment with X-rays (Preer, 1948). An analysis of the mechanisms of transformation leads to the conclusion that the determinant of killing ability (kappa), like the killing agent itself, is particulate. Preer's studies of the production of sensitive cells during rapid growth are consistent with the assumptions (1) that a single particle of kappa is sufficient to allow a cell to develop the killer characteristic and (2) that the loss of killing ability during rapid growth is due to a differential reproduction of cells and particles which results in the dilution of the particles. These studies also permitted an estimate of the number of kappa particles normally carried in a killer cell. Similarly, Preer's study of X-ray-induced transformation suggested that kappa is exponentially inactivated and that a single viable kappa particle, if given the opportunity to multiply, is sufficient to confer killing ability on a cell. These studies strongly supported the interpretation that the killer trait is determined by a particulate element whose multiplication is not precisely correlated with cell division and whose segregation can result in the formation of cells which entirely lack such particles and which, as a consequence, manifest different hereditary traits.

Further information concerning the physical basis of the killer traits emerged from breeding experiments. Sonneborn (1947) showed that when killer cells were crossed with sensitive cells derived from killers, the traits strictly followed the cytoplasm; at ordinary conjugation the exconjugant derived from a killer cell produced a clone of killer cells and the exconjugant derived from the sensiitve cell produced a clone of sensitive cells, in spite of the genic equivalence assured by nuclear exchange. When cytoplasmic exchange, as well as nuclear exchange was induced at conjugation, both exconjugants produced clones of killer cells. Hence, the particles responsible for the killer character are located in the cytoplasm. Since these particles were considered to be self-perpetuating, visualization of such particles in the cytoplasm by means of DNA-specific stains seemed a reasonable possibility, particularly since Preer's X-ray inactivation studies had suggested particle sizes near those for bacteria. Such particles were in fact demonstrated (Figs. 24, 25) in approximately the sizes and numbers predicted from indirect analysis (Preer, 1950).

A few of the remaining studies on the killer traits may be summarized briefly. Kappa is not produced by the nucleus but requires a gene, K, for its maintenance (Sonneborn, 1947); even the amount of kappa occurring in the cytoplasm under standard conditions is strictly regulated by the nuclear constitution (Chao, 1953), but no nuclear constitution known is capable of inducing kappa in the absence of pre-existing kappa. The kappa particles found in strains of different origin show significant differences in their properties (Siegel and Preer, 1957), and even within a single stock diverse kinds of kappa have been found which apparently have arisen by a mutationlike process (Dippell, 1950). In some stocks of variety 8 (one of the several "genetic species" of *P. aurelia*) kappalike bodies, designated as mu particles, are found; cells carrying these particles

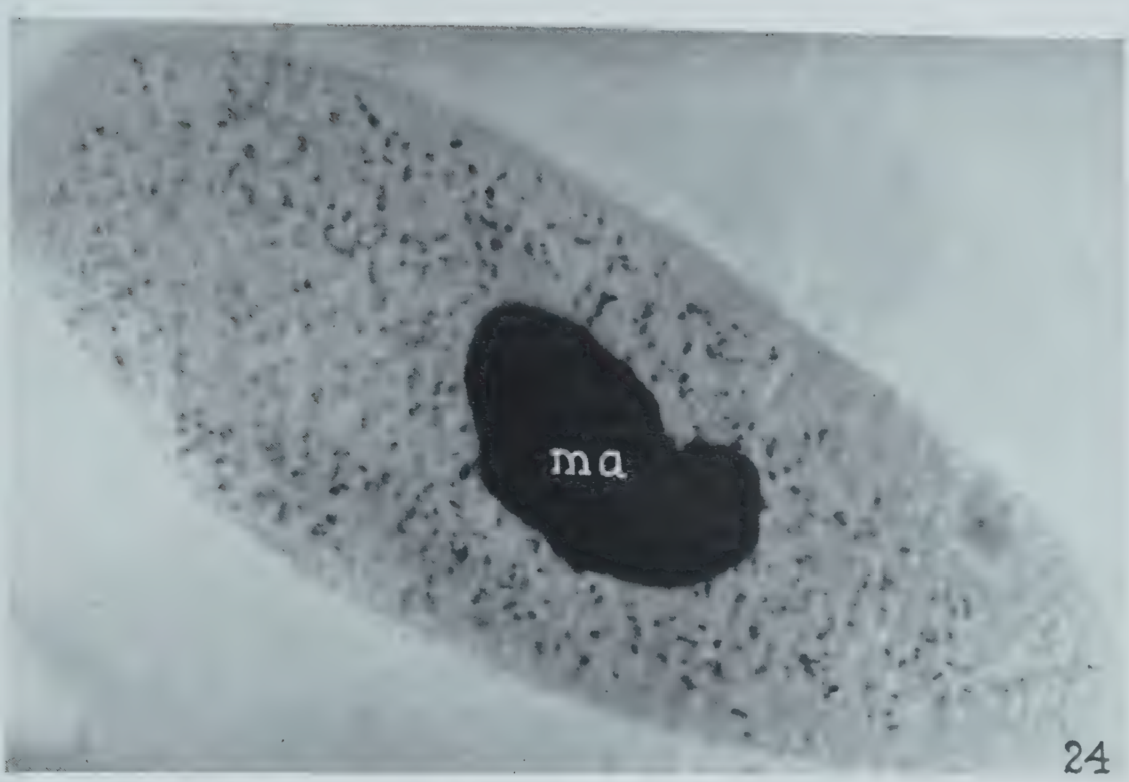


FIG. 24. Killer *Paramecium aurelia* (stock G variety 2) showing kappa. Kappa appears as small dark particles in the cytoplasm. The large dark body (*ma*) is the macronucleus. Hydrolyzed Giemsa preparation. Magnification: about ×1,000. Photomicrograph kindly supplied by Dr. R. V. Dippell (unpublished).

do not release a toxin into the medium, but kill other cells through contact at conjugation (Levine, 1953; Siegel, 1953). Even in some of the original killer stocks mutant kappas have arisen which not only fail to release a toxin, but are not known to kill other cells under any condition (Hanson, 1954). These variant strains of cytoplasmic particles have been useful in clarifying the relationship between the particles and the toxin they produce; all kappas which produce killing particles are associated with refractile bodies (Fig. 25), whereas cells with nonkilling kappas lack such elements (Preer *et al.*, 1953); from this and other evidence Preer suggests that the refractile bodies may in fact be the killing agents. Under certain circumstances kappa particles can be transferred by cell-free extracts to sensitive cells of the proper type and induce their transformation into killers (Sonneborn, 1948; Tallan, 1956).

Although much remains to be learned about the killer traits, the essential facts concerning cellular variation in this system are clear. Particles with all the essential properties of genetic elements exist in the cytoplasm; they do not arise *de novo*; they normally reproduce according to their own kind; they occasionally undergo mutational changes. They divide, however, without precise coordination with the nuclear apparatus

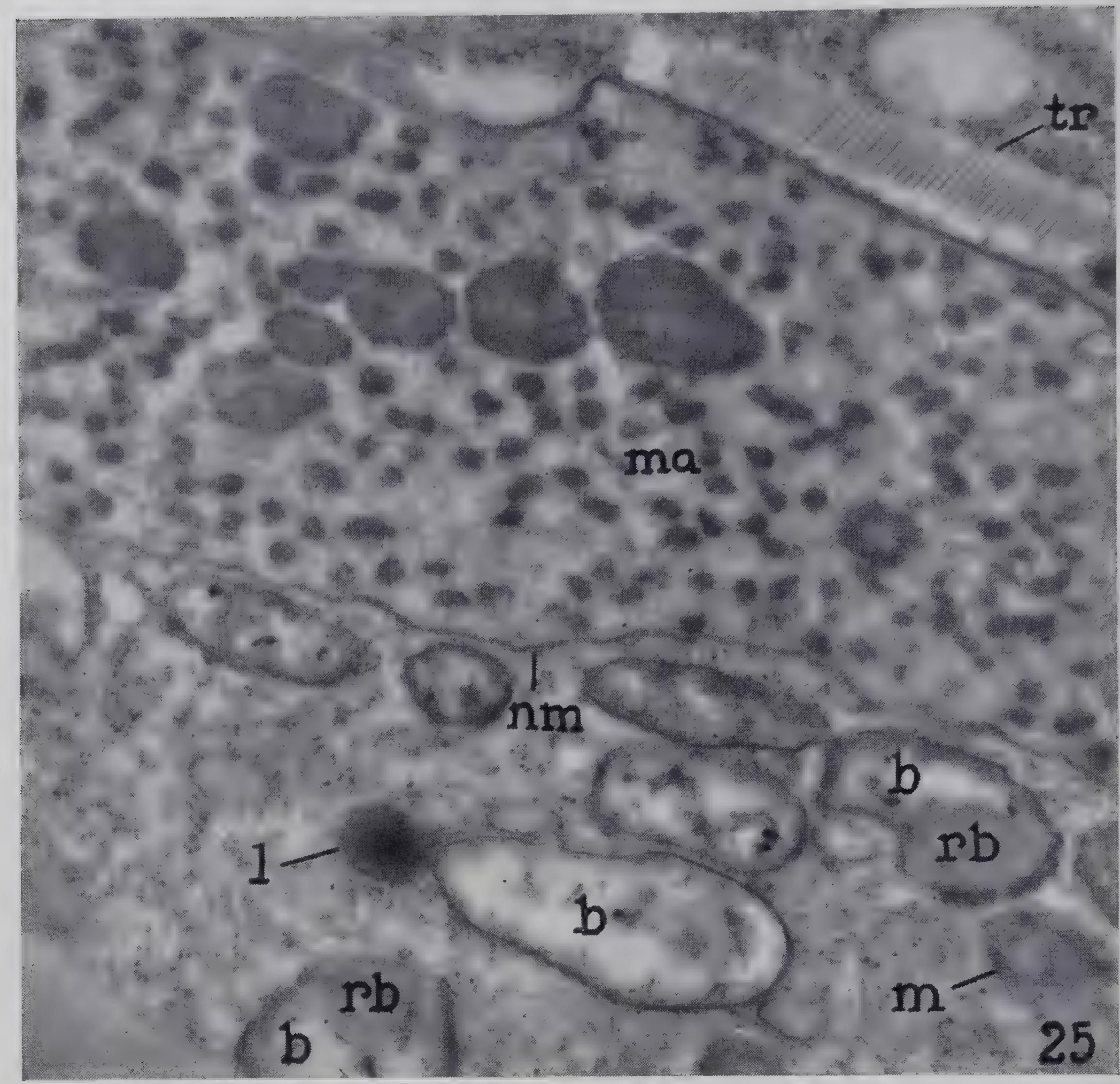


FIG. 25. Electron micrograph of section of *Paramecium aurelia* killer stock 51 showing numerous kappa bodies adjacent to the macronucleus (*ma*). Each kappa body is surrounded by a double membrane. Note the two types of kappa bodies: "brights" (*b*), containing a refractile body (*rb*), and "non-brights," which appear much denser. In the macronucleus (*ma*) the small dense granules represent chromatin bodies, the larger dense bodies represent nucleoli. A double nuclear membrane (*nm*) surrounds the macronucleus; *m*, mitochondrion; *l*, lipid body; *tr*, internally discharged trichocyst. Magnification: ×20,500. Electron micrograph taken by Dr. Keith R. Porter. (Dippell, 1958.)

and their rates of division are capable of extrinsic control. Given these properties, cytoplasmic particles can provide considerable intraclonal variation, even when the nuclear constitution remains unaltered; chance or directed loss of particles can result in permanently altered cell types. These characteristics of kappa particles are not in question, but considerable discussion has been aroused concerning what to call such particles (Lederberg, 1952) and regarding the significance and distribution of particles of this nature in other organisms.

In evaluating the significance of cytoplasmic elements, and particularly cytoplasmic elements with some degree of autonomy, the question arises as to whether these elements have intrinsic or extrinsic origins. Since their origins may be lost in distant evolutionary history, the question cannot be answered directly. Instead one must ask more limited questions, answers to which can be obtained: Can the elements reproduce outside the cell? Can they be transmitted between organisms by infection? Do they resemble other biological entities generally considered to be organisms? Do they control "normal" cellular characteristics? etc. But the answers to these questions may not permit a clear answer to the first question.

A failure, as in the case of kappa, to demonstrate *in vitro* multiplication may reflect only technical difficulties; many generally accepted parasitic organisms have not yet been grown apart from their hosts. Moreover, if mitochondria or plastids or nuclei are eventually cultivated in cell-free culture, we will scarcely conclude that they are "foreign" organisms or modify greatly our evaluation of their roles in the cell. Similarly, the demonstration of infectivity is of little value in establishing the parasitic nature of the infective agents, since almost any normal hereditary characteristic in certain bacteria can be transmitted by cell-free extracts. The remarkable degree of integration of certain bacterial viruses in the bacterial genetic system even raises a problem of interpretation regarding the origins and functional significance of these "parasites," or "semigametes." The removal of cellular constituents by treatment with antibiotics or other agents without destroying the viability of the cell does not prove that these constituents are invaders from the outside; basal granules in flagellates, plastids in certain plants, and even parts of chromosomes have been so removed.

Attempts to establish relationship between intracellular entities and independent organisms are similarly likely to yield equivocal results, since parasites are notoriously modified by the conditions of their existence. Kappa is too large, by definition, to be a virus; unfixed particles may range in size up to 10 μ in length (Preer and Stark, 1953). It shows few affinities with the symbiotic algae found in other protozoa, aside from a general similarity in the numbers found in a cell (Altenburg, 1948). If its present structure indicates homologies with other kinds of organisms (Fig. 25), the most plausible candidates are rickettsias, bacteria, or fungi; but the evidence for such relationships is not compelling.

Finally we may consider the place of kappa particles in the economy of the paramecia; can killer paramecia be considered to be afflicted with a pathogen? On this matter also only inconclusive considerations can be set forth. Certainly in the laboratory, killer cells have great selective advantages over sensitive cells grown in the same containers; whether similar advantages accrue to killers in nature has not been established. On the other hand, killers almost certainly pay a price for their armament, as is suggested by the higher respiratory rate of killers in variety 4 (Simonsen and van Wagtendonk, 1952), but this is probably true for any organism that produces a complex antibiotic and does not prove that killers are infected with a parasite.

These considerations leave in doubt the origin of kappa particles. As Sonneborn (1950b) points out, regardless of its ultimate origin, kappa must now be viewed as an element fully integrated into the economy of the paramecia, determining hereditary traits whose selective advantages and disadvantages, like those for other traits, depend on the specific environmental variables encountered. Even so, the general biological significance of kappa particles must be limited; particles of this complexity and with these properties are seldom found outside the ciliates. Because of its uniqueness, its value lies not so much in its special characteristics as in its use as a model system, illustrating the value of indirect analytical procedures in clarifying the behavior of cellular particulates. Particles endowed with genetic continuity, but much simpler in structure and smaller in size, may be understood by the application of similar techniques.

B. Variations Dependent on Alternative Cytoplasmic States

A second extensively studied system of cellular variation in the ciliates is that involving antigenic differences arising within a clone (Sonneborn, 1947, 1950b; Beale, 1954, 1957). Under certain growth conditions, the vegetative progeny from a single paramecium will manifest uniform responses (immobilization) when exposed to antiserum developed against the clone. Under other circumstances, however, the clone can be induced to produce variant subclones with different antigenic specificities. These changes do not represent temporary responses to environmental conditions, for once induced, the new antigenic characteristics may be maintained for long periods of time. Unlike the killer to sensitive transformations discussed above, however, these changes are reversible. This fact immediately suggests difficulties in interpreting the antigenic system by analogy with the killer system; if the antigens are determined by self-reproducing particles, cellular transformations cannot be explained by the loss of such particles, but only by changes in the relative frequencies of different kinds of particles. The inadequacy of even this interpretation is borne out by other observations to be mentioned below.

Some of the conditions capable of inducing antigenic transformation are treatment with sublethal concentrations of homologous antiserum, treatment with certain enzymes, and treatment by variation of a number of normal environmental factors (food supply, temperature, etc.) either singly or in combination. Although the ease and the precise conditions for transformation vary in different strains and varieties (genetic species), all strains studied have been found capable of differentiating into several different types and of maintaining different types in a common environment for a considerable length of time. The most extreme example of multiple potentialities is provided by a strain studied by Margolin (1956b) which was shown to possess a repertoire of at least twelve distinct antigenic types.

Considerable information bearing on the physical basis of these variations has been accumulated, but this information does not yet yield a completely satisfactory understanding of either the perpetuation or the transformation of antigenic types. Detailed critiques of the available evidence are found in recent reviews by Beale (1954, 1957), and only a few of the major generalizations will be noted here.

(1) *The antigenic specificities and potentialities are determined by nuclear genes.* This interpretation derives from analyses of differences observed between clones of different origin. One clone may be capable of producing an antigenic type which is not produced by another clone under any known laboratory condition; these differences in potentiality segregate as if due to single gene differences. Similarly, one clone may produce an antigenic type similar to, but distinguishable from, the corresponding type in another clone; these differences in specificity also segregate in crosses as if due to single gene differences. This nuclear localization of specificity further distinguishes the antigenic system of cellular variation from the killer system, where the specificity is cytoplasmically determined.

(2) *The cytoplasm determines which of the several genetically determined potentialities will be realized.* This interpretation is based on crosses between clones of the same genetic constitution which at the moment of crossing are manifesting different antigenic types. In the absence of cytoplasmic exchange the conjugating cells separate with identical nuclear complements (whether or not they are different prior to conjugation), but the antigenic differences are usually maintained unchanged. In contrast to this situation, conjugation accompanied by cytoplasmic exchange results in the formation of identical exconjugants; which of the parental antigenic types is expressed depends in part on the conditions accompanying conjugation. Hence, the perpetuation of particular antigenic types through sexual reorganization, and presumably through vegetative reproduction, is due at least in part to a cytoplasmic

system which selects one of the nuclear potentialities for expression. In the absence of a clear understanding of the nature of this cytoplasmic system, it is described simply as a series of "cytoplasmic states." These cytoplasmic states have a number of remarkable properties.

(3) *A particular cytoplasmic state will ordinarily permit the expression of only one nuclear potentiality.* The chief qualification required for this statement is that antigenic potentialities controlled by allelic genes are regularly expressed together, even if the antigens are serologically distinct. Only one example is known (Margolin, 1956a) of the persistent expression of traits controlled by nonallelic genes. This specificity of cytoplasmic states in their relations to nuclear genes would not, of course, be demonstrable if the cytoplasmic states were not mutually exclusive.

(4) *The characteristics of a cytoplasmic state are influenced by the nuclear genes with whose expression it is associated.* Corresponding cytoplasmic states in different stocks have been found to differ in several respects, particularly in the environmental conditions required for greatest stability. In crosses these characteristics of the cytoplasmic states segregate as if due to the segregation of nuclear genes, particularly those nuclear genes whose expression they control. In the most extreme example, the segregation of a "null allele" is accompanied by the loss of its corresponding cytoplasmic state and the establishment of a cytoplasmic state whose nuclear analog is present.

These observations make clear that the cytoplasmic system is not to be viewed as an autonomous system divorced from the nuclear gene with which it is associated, but that the nuclear genes participate actively and continuously in the maintenance of the cytoplasmic state. Specific nuclear activity is required for the perpetuation of the cytoplasmic state as unequivocally as a particular cytoplasmic state is required for the manifestation of a particular gene. The nucleus and the cytoplasm cooperate in a single system of perpetuation, and the distinction between the state of the cytoplasm and the state of the nucleus is to a large extent an accident of analytical procedures. Although superficially different, the pattern of cellular variation of antigenic types resembles in many important respects the next system of cellular variation to be discussed.

C. Variations Dependent on Alternative Nuclear States

A third system of intraclonal variation in the ciliates is that involving mating-type differentiation in forms showing "karyonidal inheritance." Before discussing the mechanisms involved in these cellular alterations, the system of breeding relations in the ciliates concerned must be briefly reviewed (Sonneborn, 1947, 1957; Beale, 1954). The "taxonomic species" in many ciliates consists of a group of effectively isolated "genetic species," designated as varieties or "syngens," within which genetic recombination may occur, but between which genetic exchange is severely restricted or impossible. In *Paramecium aurelia* at least twelve varieties are now known (Sonneborn, 1957); six have been described in *P. bursaria* (Jennings, 1939; Jennings and Opitz, 1944; Chen, 1946a) and nine in *Tetrahymena pyriformis* (Gruchy, 1955; Elliott and Hayes, 1955). Two or more physiological types (mating types) occur within each variety and conjugation normally occurs between cells of different types. The number of types within a variety differs; those in *P. aurelia* contain two mating types; those in *P. bursaria* may have as many as eight; those in *T. pyriformis* have been reported to contain from two to eleven mating types.

The details of mating type determination vary in the different species and even in different varieties of the same species, but the differences can be interpreted as variations on a single theme, the central feature in each being nuclear differentiation or "karyonidal inheritance." The simplest system of karyonidal inheritance is that found in the so-called group A varieties of *P. aurelia* (varieties 1, 3, and 5, for example). The major characteristics of this system are the following (Sonneborn, 1947; Nanney, 1954):

(1) *Cells with precisely the same genetic constitution may manifest different mating types.* The basis for this conclusion is the regular occurrence of mating type diversities among the progeny of a single conjugating pair. The occurrence of such diversities does not distinguish this system from those previously discussed; the unique characteristics reside in the pattern of distribution of these diverse cell types.

(2) *Differences in mating types observed in clones of identical genotype are determined by differences in the macronuclei.* This interpretation is based on the distribution of mating types among the progeny of single exconjugant (or exautogamous) cells. Ordinarily any diversities which will appear, do so at the time of the first division following conjugation. Since the two macronuclei formed in each exconjugant are separated at this cell division, the distribution of mating-type differences is precisely correlated with the distribution of new macronuclei. Because of the special events occurring at this division, clones having their origins in one of the first fission products—and thus sharing the vegetative descendants of a single original macronucleus—are designated as "karyonides." Cells within a karyonide are usually alike in their mating types, but the cells in sister karyonides, derived from the same exconjugant and having nuclei derived from the same synkaryon, are no more often alike than would be expected by chance alone; moreover, no relationship between the mating type of a karyonide and that of its cytoplasmic parent has been demonstrated. The correlation between macronuclear distribution and

mating-type distribution is emphasized by observations on strains characterized by unusual macronuclear distribution. Some strains, for example, may produce not two macronuclei, but three or more in some of the cells at sexual reorganization; in these strains mating-type segregation does not stop at the first division following conjugation, but continues until all the new macronuclei are assorted. Other support for this interpretation is set forth in detail elsewhere (Sonneborn, 1947; Nanney, 1954).

(3) *Although nuclear genes do not determine the mating type of a cell, they determine its mating-type potentialities.* Genes have been demonstrated which determine whether both mating types will be possible (thus permitting karyonidal differences as described above) or only one mating type (Sonneborn, 1947). Other genes have been found which influence the relative frequencies of karyonides of the two mating types (Butzel, 1955). In principle, this role for the genes in the mating-type system is essentially similar to that of the genes in the antigenic system.

(4) *The environmental conditions prevailing at the time of conjugation influence the probability of origin of macronuclei controlling particular mating types.* Sonneborn (1947) has shown that the relative frequencies of the two mating types produced at nuclear reorganization in the strains capable of producing both mating types depend on the temperature at which conjugation occurs; in variety 1, for example, mating type I is more frequent at lower temperatures. In support of the interpretation of macronuclear differentiation as the basis for mating-type determination, he found that the "sensitive period" for temperature effects on mating-type determination is the time during which the new macronuclei are developing. Other influences on mating-type frequencies have also been observed, but these have not been fully characterized.

The system of mating-type determination in variety 1 of *T. pyriformis* is essentially the same as that in the group A varieties of *P. aurelia* (Nanney, 1956). The chief difference lies in the fact that several, instead of only two, alternative mating types are possible, but—as in *P. aurelia*—mating-type differences regularly segregate at the first postzygotic cell division when the macronuclei are assorted. Again the nuclear genes control a spectrum of potentialities and determine the relative frequencies of expression of the different mating types; they do not, however, prescribe which of the potentialities will be expressed in any particular karyonide. Environmental effects on mating-type determination in this system have not been adequately explored.

Another system showing the essential features of the previous system, but including some new elements, is that observed in the group B varieties (2, 4, 6, 8, and others) of *P. aurelia* (Sonneborn, 1947, 1957; Nanney, 1954). As in the group A system, cells with the same genetic constitution may manifest different mating types, but this is demonstrated in a different way. Instead of mating-type diversities arising regularly at the first postzygotic cell division, sister karyonides from the same exconjugant are usually of the same mating type, but exconjugants from the same mating pair usually produce progeny of different mating types. Hence, cells with the same genotype (exconjugants in this case) manifest different mating types. Since the cells uniting in conjugation are of different mating types, and since they yield progeny of different mating types, some mechanism to assure the maintenance of mating type through sexual reorganization must be operating. This mechanism does not depend primarily on nuclear genes, for these become equivalent at conjugation; it does not require the continuity of macronuclear material, for the macronucleus is destroyed following conjugation. Hence, the mechanism for perpetuation must include a cytoplasmic component. This conclusion is supported by the observation that conjugation associated with massive cytoplasmic exchange regularly results in the production of identical mating types in the two exconjugants. The relative frequencies of pairs producing the two mating types when cytoplasmic exchange is induced depend on the temperature at which conjugation occurs.

In spite of this direct evidence for cytoplasmic control of mating-type perpetuation during conjugation in the group B system, equally strong evidence for macronuclear differentiation, similar to that in the A system is available. The most elegant demonstration of this fact is found in Sonneborn's (1954a) study of macronuclear regeneration in variety 4. The major conclusions from these studies can be summarized without recounting all the details of the experiments. By special techniques Sonneborn was able to develop cells containing two kinds of macronuclear material, fragments of old macronuclei from a cell of mating type VII and new macronuclei which had been exposed to cytoplasm under conditions suitable for the development of mating type VIII. These two types of macronuclear material were then induced to assort during vegetative growth, and the mating types of the various lineages were correlated with the type of macronuclear material which they received. In each instance the assortment of the two kinds of material was associated with the assortment of mating types. Moreover, at the next sexual reorganization, when the state of the cytoplasm could be tested again, the cytoplasmic conditions normally associated with the particular mating types were observed. Hence, although the cytoplasm controls the direction of macronuclear differentiation, once the macronucleus is established it imposes its control on the cytoplasm. The perpetuation of mating types in the group B varieties must, therefore, be viewed as a closed cycle of nucleocytoplasmic interactions.

The third variation on the theme of nuclear differentiation is found in the system of mating-type determination in *P. bursaria* (Jennings, 1942; Sonneborn, 1947, 1957). An essentially similar system has recently been reported for variety 2 of *T. pyriformis* (Hurst, 1957). Unlike the two systems mentioned above, mating-type diversities do not regularly appear among the progeny of single conjugating pairs; sister exconjugants usually produce clones of the same mating type, and usually of the same mating type as one of the parents. Nevertheless, diversities do arise occasionally, and these may be segregated at the first fission following conjugation. Though rare, these exceptional pairs occur too often to be explained readily as due to mutation. Moreover, the relative frequencies of pairs showing the two parental mating types, or of other nonparental mating types, do not find a ready and simple explanation in genetic recombination. An explanation based on analogy with the above systems appears, therefore, to be most promising.

When cytoplasmic exchange is induced between mating cells in the group B varieties of *P. aurelia,* results essentially like those in *P. bursaria* are obtained, i.e., pair members tend to be alike in mating type, but some pairs manifest one parental mating type and others manifest another parental mating type. Hence, if cytoplasmic exchange regularly occurs at conjugation in *P. bursaria* and in variety 2 of *T. pyriformis,* the results are in agreement with the operation of a group B system of mating-type determination, i.e., a system in which the cytoplasm determines the course of nuclear differentiation. This interpretation is supported by evidence for cytoplasmic exchange at conjugation in *P. bursaria* (Harrison and Fowler, 1945; Chen, 1946b); but rigorous demonstration of cytoplasmic exchange in variety 2 of *T. pyriformis* has not yet been obtained.

These observations on mating-type differentiation in *Paramecium* and *Tetrahymena* establish nuclear differentiation as an important factor in hereditary intraclonal variation and provide considerable insight into the factors, environmental, cytoplasmic, and genic, which influence the course of nuclear differentiation. They do not yet, however, provide a satisfactory understanding of the nature of the nuclear differences. The alterations responsible for mating-type determination may be alterations of a sort possible only in the complex and poorly understood ciliate macronucleus, or they may be alterations similar in kind to those which have been demonstrated during embryonic development (King and Briggs, 1956) or those responsible for antigenic variation in *Salmonella* (Lederberg and Iino, 1956).

D. Age-dependent Variations

Some of the least understood and, perhaps for this reason, most interesting of cellular variations in the ciliates are those alterations which follow each other in regular sequence following the origin of a clone at sexual reorganization. The analysis of such changes is made difficult by the usual obliteration and reinitiation of the pattern at a subsequent sexual reorganization. The occurrence of patterned sequential changes in ciliates was first noted by Maupas (1889), but our attention will be focused on some of the modern studies. One such study is that of Jennings (1945) with *Paramecium bursaria.*

Immediately following conjugation, cells of *P. bursaria* enter a period of sexual immaturity during which they are incapable of conjugation. This period lasts for a variable length of time, but may extend for a period of several years and for hundreds of cell generations. Hence, the condition of immaturity is "inherited" for long periods of vegetative growth. Immaturity is followed by a relatively short transition stage during which cells gradually acquire the ability to mate with mating types other than their own; this transition period is sometimes designated as "adolescence." It is followed by a period of full maturity which again may last for very long periods of time. Eventually, however, clones gradually enter a period of "senility." At this time several changes are observed. The stability of the mating-type system may be altered and a clone may become capable of "self-differentiation," i.e., capable of giving rise to sublines of a different mating type than that maintained previously. More importantly, however, the cells gradually lose the ability to survive conjugation until eventually all cells entering a sexual reorganization die. These observations, therefore, outline a pattern of clonal alterations making up a complete life cycle and including immaturity, adolescence, maturity, and senility.

Similar observations have been made by Sonneborn (1954b) for *P. aurelia.* Again immediately following conjugation, cells may enter a period of sexual immaturity. In some strains this period of immaturity lasts for a relatively long time (i.e., dozens of cell divisions); in others the period is very short and in still others it may be eliminated entirely. This stage is followed by a period of maturity, possibly preceded by a short transition stage. In the absence of conjugation the cells gradually acquire the abiilty to undergo autogamy (self-fertilization), which normally reinitiates the cycle. When both conjugation and autogamy are prevented, however, the cells continue to divide and other alterations become apparent. Eventually the cells begin to lose their ability to undergo autogamy, or, if they undergo autogamy, to survive the process. Similarly, a time series of crosses with these aged clones yields progressively fewer survivors. Associated with the death which accompanies sexual reorganization are cytological abnormalities, and the survivors may acquire genetic alterations which behave like chromosome aberrations. In this period of decline the cells grow at progressively slower rates until eventually they divide not at all and the clone dies. These alterations may not occur at precisely the same time in sublines of the same clone, but they are highly correlated and even with constant selection vigorous clones cannot be maintained.

This pattern of life cycle including at its terminus a period of senility and death may not be universal among the ciliates. Some, like *Tetrahymena,* have been maintained in mass cultures without sexual reorganization for many years with no signs of decline in vigor (see Nanney, 1959). Such forms may show a part of the life cycle—periods of immaturity, adolescence, and maturity—without the final stages, just as some strains of *P. aurelia* omit the stages of immaturity and adolescence. On the other hand, Sonneborn (1957) has suggested that the major distinction between forms like *P. aurelia* and forms like *T. pyriformis* lies in the relative length of the life cycle, and he proposes that the amicronucleate strains of *Tetrahymena* are senile strains which are already dead genetically.

The mechanisms underlying the age-dependent clonal variations have thus far resisted the analytical tools available. Fauré-Fremiet (1953) has suggested that some of them may be the inevitable consequences of some special feature of ciliate organization, but the great variability in the kinds of changes and in their time courses—coupled with the demonstration of their adaptive significance (Sonneborn, 1957)—makes this interpretation unattractive. Moreover, remarkably similar phenomena have been reported in organisms of very different kinds (Jinks, 1956). One possible explanation for such alterations assumes a differential production or accumulation of material. Either some type of material is gradually depleted during vegetative growth and restored at sexual reorganization, or some critical material is gradually accumulated, in either the nucleus or the cytoplasm. The abrupt transitions between stages, where they occur, could suggest threshold effects. The demonstration of accumulated or depleted materials has not yet been accomplished, however, and no evidence associating some particular material change with some particular biological alteration has been obtained. Moreover, the extremely long growth periods through which the postulated changes occur are difficult to explain with present models.

IV. CONCLUSION

This brief summary of selected studies on ciliated protozoa includes no mention of the enormous literature on the biochemistry and nutritional physiology of the ciliates and but limited reference to cytochemical and electron microscopic studies; other aspects of the cellular biology in these forms have been similarly ignored or treated superficially. Instead, an attempt has been made to focus attention on problems of cellular regulation and cellular integration, a procedure which has perhaps raised more questions than have been answered. Certainly in the ciliates, and indeed in all cells, complex mechanisms exist for the coordination of cellular activities.

These mechanisms accomplish a variety of ends. Some may be considered homeostatic mechanisms which maintain a cell in a particular morphological or physiological state and return the cell to that state when it is disturbed. In some instances these homeostats operate for a limited time, but in other cases they persist over long periods of multiplication and must be considered "hereditary" mechanisms. Chromosome replication and mitosis are the most familiar homeostatic devices, operating to maintain intact the reservoir of genetic information; these are the "true" genetic mechanisms. Not all cellular properties which are perpetuated can, however, be directly referred to the primary genetic material. The serotype and mating-type systems reviewed in previous sections provide evidence for auxiliary mechanisms which choose particular specificities from the library and perpetuate their expression. These supplementary devices have been termed "epigenetic systems" (Ephrussi, 1958; Nanney, 1958a, b) to distinguish them from genetic systems proper and to emphasize their significance in development.

Epigenetic systems can be called upon, however, to perform many different functions. They are conceived as the devices responsible for rhythmic patterns of activity, for directed sequential changes in cellular properties, and for specific responses of cells to environmental variables. Beyond establishing their existence, the work on ciliates has not gone far toward providing an understanding of how these mechanisms operate. Most of the work has, in fact, been directed toward an understanding of the epigenetic homeostats.

The studies on "hereditary," but "nongenetic," cellular differences raise many problems. They imply that cells with the same genotypes, existing and reproducing in the same environment, may manifest different traits. They suggest that a cell may store information about its past experiences in forms other than nucleotide sequences. They indicate that the time-honoured operational distinction between genetic and nongenetic cellular traits is of limited validity. If differences disappear when different cell types are cultivated for long periods in a common environment, they are not hereditary and not genetic; but if the differences persist, they may be controlled by either genetic or epigenetic homeostats. What operations, then, can provide distinctions between control by genetic and epigenetic systems? Unfortunately, no single operation—not even breeding analysis—appears adequate at the present time and no combination of operations is uniformly reliable. Fortunately the investigator employing ciliates is richly supplied with experimental resources. He can modify the extracellular environment; he can induce mating of differentiated cells; he can in some cases control the cytoplasmic and nuclear contributions of the parents in crosses; he has a variety of special cytogenetic processes at hand which are capable of manipulation; he has a vast supply of organisms which differ in the details of their behavior and cross-illuminate each other in comparative studies. Even so, distinctions between genetic and epigenetic systems are not always easy.

Once the distinctions have been made, an even more difficult problem remains, that of understanding the mode of operation of the homeostats. Thus far some success has been achieved in describing epigenetic systems, in demonstrating that their specificities are dependent on the genetic reservoir, and in showing that they involve feedback systems with various cellular locations and of various stabilities. The available information does not yet permit an analysis of epigenetic systems in molecular terms, and this must be considered a major outstanding task. The work accomplished thus far poses questions and provides a frame of reference within which these further studies will be profitable.

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Postscript

Since the text was prepared, several studies have been published which modify or extend the evaluations set forth. In some cases the papers appeared in time to be included in the reference section, but not in time for adequate textual modification. These include the references on "epigenetics" (Ephrussi, 1958; Nanney, 1958a, 1958b), on ageing (Nanney, 1959), on trichocysts (Preer, 1959), and most especially Sonneborn's (1959) exhaustive review on kappa and related particles. Other papers related to the problems discussed should also be cited. These include studies on: (1) *Cortical Organization.* Bradfield, J. R. G. (1955). *Symp. Soc. Exptl. Biol.* **9,** 306. Ehret, C. F., and Powers, E. L. (1959). *Intern. Rev. Cytol.* **8,** 97. Noirot-Timotheé, C. (1958). *Compt. rend. Acad. Sci.* **246,** 2293. Roth, L. E. (1958). *J. Ultrastructure Res.* **1,** 224. (2) *Macronuclear Organization.* Allen, S. L., and Nanney, D. L. (1958). *Am. Naturalist* **92,** 139. Fauré-Fremiet, E. (1957). *J. Protozool.* **4,** 7. Fauré-Fremiet, E., Rouiller, C., and Gauchery, M. (1956). *Exptl. Cell Res.* **12,** 135. Gall, J. G. (1959). *J. Biophys.*

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CHAPTER 4

Intracellular Parasitism and Symbiosis

By WILLIAM TRAGER

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I. INTRODUCTION

The cells of plants and animals are often invaded by parasitic microorganisms. Some of these intracellular parasites have attracted special attention because they are agents of disease in man or in animals or plants of economic value. One need only mention the bacillus of leprosy, the rickettsiae of typhus fever, the protozoa of human malaria, the pox viruses, the rust fungi, the slime mold which produces clubroot of cabbage. It is often forgotten that certain intracellular microorganisms are essential to the well-being of their host. The root nodule bacteria of leguminous plants and the symbiotic bacteria and yeasts of many kinds of insects live in such a mutualistic association with their host. The legume bacteria supply more nitrogen to cultivated plants than does any other single source (Virtanen, 1947).

To the general biologist and the cell physiologist the intracellular microorganisms are of particular interest quite apart from their medical and economic importance. They represent an extreme of ecological specialization—one organism living not merely within the body cavities or fluids of another but actually within the living cells of the host. What are the precise physiological relationships between host cell and parasite? What special nutrients must the parasite obtain preformed from its host? How do parasites penetrate the cell membrane of the host without disrupting it? How do the parasites influence the metabolic activities of the host cell? How is it, for example, that penetration of a cell by the protozoan parasite *Leucocytozoon,* or by the slime mold *Plasmodiophora,* is followed by gross enlargement of the host cell and its nucleus? How does it come about that the cells of the root nodules of leguminous plants produce hemoglobin only when they are infected by symbiotic bacteria? We must admit at once that the answers to most of these questions and to many others of equal significance are not yet available. The future researches from which one may hope they will be obtained must be based on the large body of facts already known about intracellular microorganisms, and on some working hypotheses. These facts and hypotheses will form the substance of the present chapter.

A. Origin of the Intracellular Mode of Life

Intracellular parasitism occurs among so many different groups of small organisms that it must have arisen at many times in various ways and for different reasons. Some virologists derive the viruses from self-reproducing units of cells—genes, plasmagenes, broken portions of chromosomes, etc. There is much evidence to support this view (Luria, 1953). The viruses, however, are not a homogeneous taxonomic group. The larger viruses may well have been derived by biochemical regressive evolution (Lwoff, 1936), perhaps from protozoa, via intermediate structures such as kappa of *Paramecium* or *Anaplasma* of cattle.

Certainly among the intracellular parasites other than viruses—the bacteria, rickettsiae, and protozoa—the loss of independent metabolic activities appears to have accompanied the specialization for intracellular parasitism. All the evidence would indicate that the intracellular parasitic forms have evolved from less specialized parasites and these in turn from free-living species. The acid-fast bacilli furnish an example of such a series. As one goes from the saprophytic mycobacteria to the "atypical pathogens," then to the tubercle bacilli, to Johne's bacillus, and finally to the bacilli of murine and human leprosy, one finds that the trend to slower growth rates, increased fastidiousness of growth requirements, and ultimately noncultivability, is accompanied by stepwise limitations in oxidative capacity (Hanks, 1954a). The tubercle bacillus, a facultative intracellular parasite, attacks and kills cells to form an extracellular medium in which it can multiply. The acute tubercular lesion is typified by tissue destruction. The leprosy bacillus gets ingested by the host cell and multiplies slowly within it. The severe lesion of leprosy is characterized by a granuloma (Muir, 1957).

The hemoflagellate protozoa similarly show a gradual series from parasites of the intestinal lumen of insects to obligate intracellular forms. The parasites which cause visceral leishmaniasis exist in their mammalian host as rounded intracellular bodies, but in the gut of their sand fly vector they multiply extracellularly. There is reason to think that this difference in habitat may be associated with additional nutritional requirements imposed by the higher body temperature of the mammalian host, a matter we shall consider in more detail later on.

In the protozoan subphylum Sporozoa, all the known species are parasitic. Some, as malaria parasites, are intracellular, whereas others, as the gregarines of the alimentary tract of insects, are extracellular. It is a remarkable fact, however, that many of these gregarines spend considerable portions of their life cycle attached to a host cell by a special organ, the epimerite, through which they may absorb nutriment. In their nutritional physiology such parasites are perhaps not very different from intracellular parasites. The same may be true of the various poorly understood organisms associated with the external surface of red blood cells—organisms of the genera *Bartonella, Grahamella, Eperythrozoon.* Only two species of this group, *Bartonella bacilliformis* of rats (Peters and Wigand, 1955) and a *Grahamella* from mice (Tyzzer, 1941) have been cultured *in vitro,* so that the nutritional requirements of most of them may be as complex as those of the intracellular rickettsiae they so closely resemble.

B. Definition of an Obligate Intracellular Parasite

It is evident that in the study of intracellular parasitism, as everywhere in nature, definitions are not easily arrived at. There are microorganisms which live on the surface of other cells but seem never to enter them; there are others which are sometimes on and sometimes in; still others which lie mainly outside the cell but appear to derive their nutriment from within it; others which regularly pass one portion of a life cycle intracellularly and another extracellularly; others which may develop intra- or extracellularly depending on environmental conditions; others which develop only intracellularly but can survive extracellularly; and finally those which always lie within a host cell. This last group is not a large one. It does not include, for example, the many viruses capable of extracellular survival during transmission from one host to another. It does include parasites such as kappa of *Paramecium,* which passes from one host cell to both daughter cells at division.

We may define an obligate intracellular parasite as a parasite which in nature grows only within a living host cell or at the expense of nutriment derived from a living host cell. This definition would exclude such facultative intracellular parasites as the tubercle bacillus. It would include the intracellular stages of parasites having a life cycle in which extracellular growth stages also occur. For example, both the erythrocytic and exoerythrocytic stages of malaria parasites are obligately intracellular; these stages, which occur in the vertebrate host, never develop extracellularly in nature. On the other hand, the stages which occur in the mosquito are extracellular (Weathersby, 1952). The definition would also include such parasites as the rust fungi of plants. These organisms develop an extracellular mycelium which, however, derives its nutrients by means of haustoria which invade the living cells of the host plant (Walker, 1950). The relationship to the host is very different from that existing with predatorlike parasitic fungi which cytolyze the host plant by means of powerful hydrolytic enzymes. In framing our definition, the term parasite has been used in its broadest sense to include not only organisms which harm the host or leave it unaffected, but also those which benefit the host. It will be useful now to differentiate between parasites in the narrower sense, as implying forms more or less pathogenic, and symbionts, the forms which enter into mutualistic association with the host. It is probably a significant fact that none of the microorganisms which fit the definition of an obligate intracellular parasite in this narrower sense has yet been cultivated *in vitro* apart from living host cells. This is as true of the leprosy bacillus as it is of rickettsiae; it is as true of malaria parasites as of pox viruses; of the rust fungi as of kappa of *Paramecium*. A number of the symbiotic microorganisms, even though they exist only intracellularly in nature, can be cultured readily; for example the yeasts of certain insects and the rhizobia of legumes. The great majority of symbionts, however, like the parasitic forms, have not been cultured.

C. Cellular Organelles and Intracellular Parasites

Intracellular microorganisms which cause disease and which can be seen with the light microscope have not as a rule been considered to be cellular organelles. Altogether similar structures having a symbiotic relationship to the host have been so considered. The pathogenic rickettsiae are generally admitted to be microorganisms, even though they produce no disease in the arthropod vector and even though they may be transmitted transovarially. Nearly identical bodies which however are restricted to an arthropod host have been claimed to be normal cellular organelles. Even large structures, morphologically and histochemically resembling in every detail yeasts and bacteria, have at times been described as cellular organelles. This has been done especially with the intracellular symbionts of insects. Despite the fact that it is becoming increasingly apparent that such organisms behave as an integral part of the host and are beneficial to it, there is nothing to be gained by calling them cellular organelles. On the other hand, it is only just to say that some bodies which have been called symbionts may be normal granular components of the cell. As their size gets smaller and as we have less knowledge about them and fewer criteria for their identification, the problem of differentiating between a parasitic microorganism and a self-duplicating cellular inclusion becomes progressively more difficult. Luria (1953) has discussed this matter in some detail in connection with the possible origin of viruses. It might even be that a number of present-day cellular organelles originated as infectious microorganisms which finally entered into a wholly stable association with the host cell. It seems likely that, just as light microscopy has shown quite clearly that the larger intracellular parasites have the properties of microorganisms (Buchner, 1953; Richards and Brooks, 1958), so electron microscopy may well reveal a basis for classifying the smaller ones as either cellular organelles or greatly regressed microorganisms. Eventual success in cultivation of intracellular parasites or symbionts apart from the living host cell is not in itself a proof of their microorganismal nature. There is no theoretical reason why conditions could not be found which would support the multiplication *in vitro* of any self-duplicating cellular entity.

II. Kinds of Intracellular Parasites

The widespread occurrence in nature of intracellular parasitism can best be emphasized by a brief consideration of the kinds of intracellular microorganisms.

A. Algae

The zoochlorellae and zooxanthellae, representatives respectively of the orders Chlorophyceae and Chrysophyceae, occur within the cells of a variety of aquatic invertebrates. Zoochlorellae typically are found in fresh-water organisms; the best-known examples are those in *Paramecium bursaria* and in *Hydra viridissima* (see Buchner, 1953). Zooxanthellae live in association with marine invertebrates, especially in coral reef communities, as in the mantle of the giant clam and in Scyphozoa, anemones, and coral (Yonge, 1944). There are some indications that these algae are beneficial to the host, but the association often is not an obligate one for either of the partners.

B. Fungi

Although a great many different kinds of fungi parasitize plants and animals, relatively few display the high degrees of specialization associated with obligate intracellular parasitism. Perhaps the simplest of these, and the least well known, are the chytrids (Phycomycetes), such as the various species of *Sphaerita* which occur frequently in protozoa (Lubinsky, 1955). *Histoplasma capsulatum* and *Coccidioides immitis* (both Fungi Imperfecti) produce disease in man and other animals. Both are characterized by a distinctive intracellular stage, yeastlike in *Histoplasma* and spherical with endospores in *Coccidioides,* which can be reproduced in culture at 37° C. only under rather special conditions (Conant, 1952).

Of the greatest interest, and incidentally of greatest economic importance, are three types of fungus parasites of plants. The first is represented by the etiologic agent of clubroot of cabbage. This organism, *Plasmodiophora brassicae* (an ameboid slime mold), develops intracellularly in the roots of cabbage as amebas or small plasmodia. It stimulates giant cell formation and hyperplasia. In 1878, when Woronin first described the life cycle of this parasite and its pathogenic effects, he remarked that a more pentrating knowledge of how the hyperplasia and hypertrophy were produced might have some bearing on the problem of cancer.

The other two types of highly specialized fungus parasites of plants are the powdery mildews and the rusts. Although these belong to two different groups of fungi (Ascomycetes and Basidiomycetes, respectively) and have very different life cycles—that of the powdery mildews being a simple one with a single host whereas that of the rusts is complex and involves at least two hosts—nevertheless the relationship between the parasite and the host plant is a similar one. In both groups an extracellular mycelium is formed which, however, depends for its nutriment on special processes, the haustoria, which penetrate inside the host cells. As is typical of all obligate intracellular parasites, the host cells invaded by the haustoria at first show no ill effects. Later nuclear enlargement appears, followed by collapse of the nucleus (Walker, 1950). Despite many attempts, none of the powdery mildews or rusts has ever been cultured *in vitro*.

C. Symbiotic Yeasts and Bacteria

The mutualistic association between leguminous plants and their root nodule bacteria (rhizobia) has been known and studied for a long time (Allen and Allen, 1954). Rhizobia are in most respects quite ordinary bacteria; their one distinctive feature is their ability to infect the roots of appropriate species of legumes with the formation of nodules and with the creation of conditions which permit the fixation of atmospheric nitrogen by the host-parasite complex. This fascinating and economically extremely important partnership will be discussed in more detail in a later section of this chapter.

Most of the other symbiotic intracellular bacteria and yeasts occur in animals, especially in insects. The classical monograph of Buchner (1953) describes in detail the bewildering variety of forms, and the amazingly complex methods of transmission from one host generation to the next, of these microorganisms. Compared to the wealth of morphological information, relatively little is known about the physiological relationships of these parasites to their hosts.

D. Pathogenic Bacteria

The bacilli of rat and human leprosy are the only well-studied pathogenic bacteria which fit our definition of an obligate intracellular parasite. We have already noted briefly how the leprosy bacilli differ from other pathogenic mycobacteria in their relationship to, and their effects on, their host cell. Facultative intracellular parasitism, on the other hand, is not uncommon among pathogenic bacteria. The tubercle bacillus, *Brucella abortus*, and perhaps especially the organism of tularemia, are good examples of such facultative intracellular parasites. *Pasteurella tularense* has been observed to be intracellular not only in its vertebrate host, but also in ticks of the genus *Ornithodoros* which probably serve as reservoirs as well as vectors of the infection (Burgdorfer and Owen, 1956).

E. Rickettsiae

Rickettsiae have been defined as small, intracellular, gram-negative, bacteria-like, pleomorphic organisms (Zinsser, 1937). They have been found in many species of insects, ticks, and other arthropods (Cowdry, 1923). In these they exhibit a remarkable degree of host specificity, they are often transmitted through the egg, and usually they neither excite a cellular response in the host nor produce any pathological effects. It is difficult to know just where to draw the line between such nonpathogenic rickettsiae and large bacterial symbionts of insects (Hertig and Wolbach, 1924). Moreover, a few species of microorganisms morphologically identical with rickettsiae grow extracellularly in their insect host—for example *Rickettsia melophagi* of the sheep kid (Hertig and Wolbach, 1924) and *R. pediculi* of the louse. The latter organism also grew extracellularly in culture preparations of surviving louse tissues (Nauck and Weyer, 1941), whereas typhus fever rickettsiae grew only within the cells of the louse tissue.

The pathogenic rickettsiae include those which produce in man the fevers of the typhus and spotted fever groups, scrub typhus, and Q fever, as well as *R. ruminantium* (Cowdry, 1925) which causes heartwater disease of cattle. All of these are obligate intracellular parasites in their invertebrate vectors as well as in their vertebrate hosts. Recently a rickettsia has been shown to be the etiologic agent of salmon-poisoning disease of dogs (Philip *et al.*, 1954). Unlike all other known rickettsiae, this one is transmitted by means of a trematode having snails and fish as its intermediate hosts.

Rickettsiae have a cellular organization similar to that of bacteria. Nuclear material has been seen in living organisms with the phase contrast microscope as well as by suitable staining (Ris and Fox, 1949). A somewhat denser central region is apparent in electron micrographs of thin sections of purified rickettsiae subjected to ether extraction (Stoker *et al.*, 1956), but sections through rickettsiae fixed within their host cells show by electron microscopy numerous scattered dense granules (Wissig *et al.*, 1956). (Fig. 1). Rickettsiae reproduce by binary fission as do bacteria (Schaechter *et. al.*, 1957).

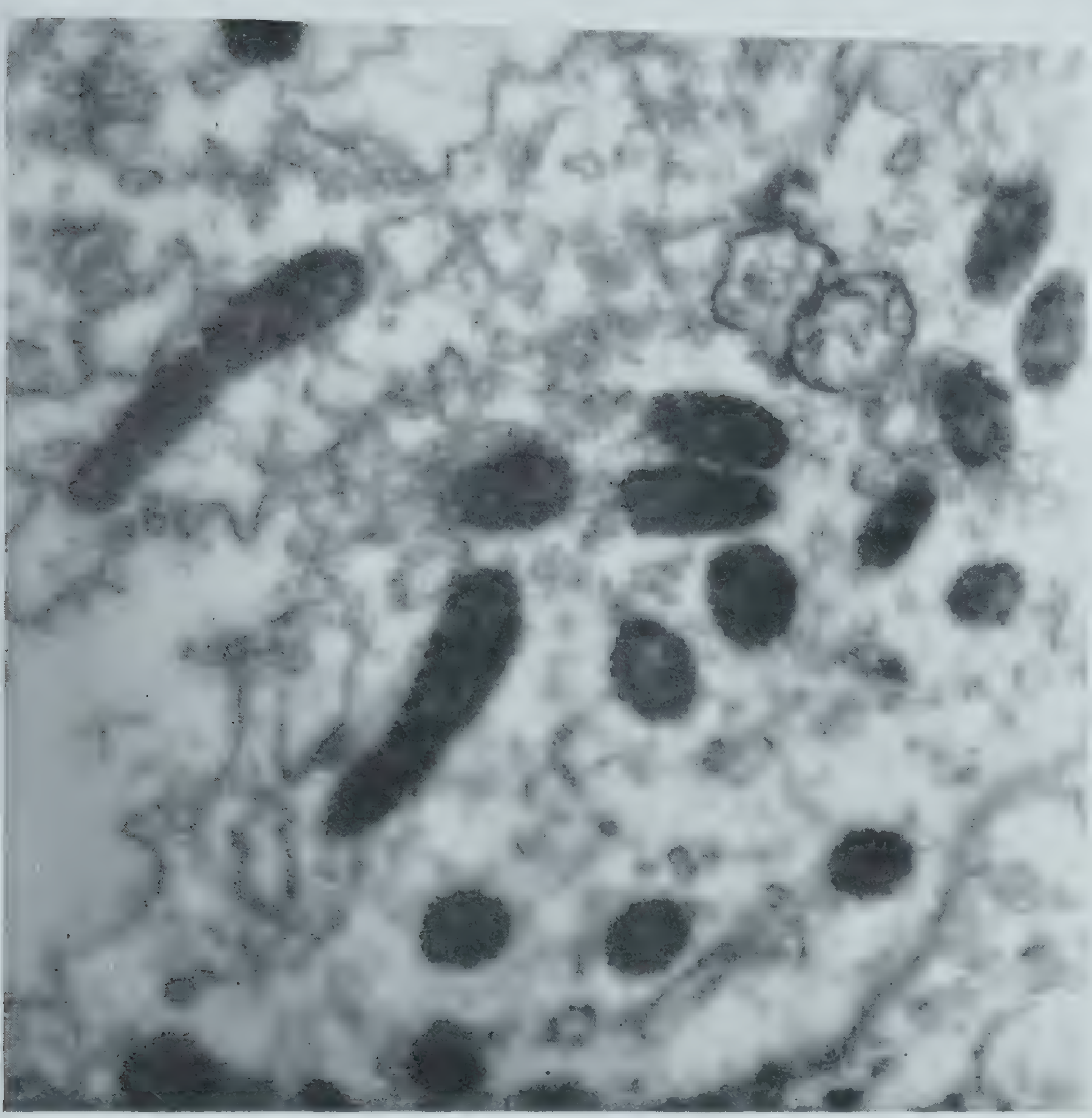


FIG. 1. Rickettsiae in a mesothelial cell. Electron micrograph of a thin section of infected yolk sac of 12-day chick embryos inoculated 6 days previously with *Rickettsia mooseri*. The rickettsiae have been cut in various planes. Note the two attached pairs of rickettsiae toward the left of the picture and at the upper right-hand region, two mitochondria (less dense than the rickettsiae). Magnification: ×30,948. From Wissig *et al.* (1956); reproduced with the kind permission of the authors and the editors of the *American Journal of Pathology*.

F. Protozoa

An entire subphylum, the Sporozoa, consists exclusively of parasitic species most of which undergo all or important parts of their development within the cells of their host (Hall, 1953). Not a single member of the Sporozoa has yet been grown continuously *in vitro* apart from its living host cell, despite the fact that the subphylum includes such important and thoroughly studied species as the malarial parasites, the coccidia of chickens and other domestic animals, the piroplasms of Texas fever and East Coast fever of cattle, and the *Nosema* which produces pebrine of silkworms, one of the first infectious entities studied by Pasteur (Vallery-Radot, 1926). Some of the Sporozoa are intracellular only in the very young stages of development, the later stages being lumen-dwelling forms often, however, attached to a host cell by a special organ, the epimerite. Other groups, such as the coccidia, undergo all their growth as intracellular parasites. Still others, as the malaria parasites, pass through intracellular cycles of asexual reproduction in the vertebrate host and an extracellular cycle of sexual reproduction in the invertebrate host.

Besides the Sporozoa, protozoa with the intracellular habit occur in the genus *Toxoplasma,* which is of uncertain taxonomic position, and in the class of flagellates, the Zoomastigophora. *Toxoplasma* is an obligate intracellular parasite which can invade a variety of cell types and which resembles some viruses in being able to infect many species of vertebrate hosts. It is the cause of toxoplasmosis, a disease of man which is now recognized to be much more widespread and important than had formerly been thought (Jacobs, 1956). *Leishmania donovani,* the agent of visceral leishmaniasis, and *Trypanosoma cruzi,* the cause of Chagas' disease, are examples of hemoflagellates which multiply in the vertebrate host only intracellularly as small ovoid bodies, the leishmania. In the insect vector they multiply extracellularly as motile flagellates.

G. Anaplasma, Kappa

The anaplasma bodies of cattle and kappa of the ciliate *Paramecium aurelia* are infectious pathogenic entities which cannot be classed with rickettsiae, bacteria, or protozoa and are certainly too large to be considered viruses. They are discussed together here only for the sake of convenience, not because they are necessarily closely related. Both may represent residual forms of intermediate nature and hence of the greatest evolutionary significance. Unfortunately, both are as yet poorly understood.

Anaplasma bodies are small, circular, basophilic structures found in erythrocytes of cattle suffering from anaplasmosis, a disease characterized by a progressive anemia (Dykstra *et al.,* 1948; Schmidt, 1956). The infection is transmitted in nature by ticks, in which a developmental cycle probably occurs, and also mechanically by tabanid flies. The anaplasma bodies are neither Howell-Jolly bodies nor degradation products. They contain deoxyribonucleic acid, ribonucleic acid, protein, and organic iron (Moulton and Christensen, 1955). Their highly specific nature has been demonstrated by the fluorescent antibody technique (Fig. 2) (Ristic, *et. al.,* 1957). Electron microscopy of thin sections of infected red cells of cattle

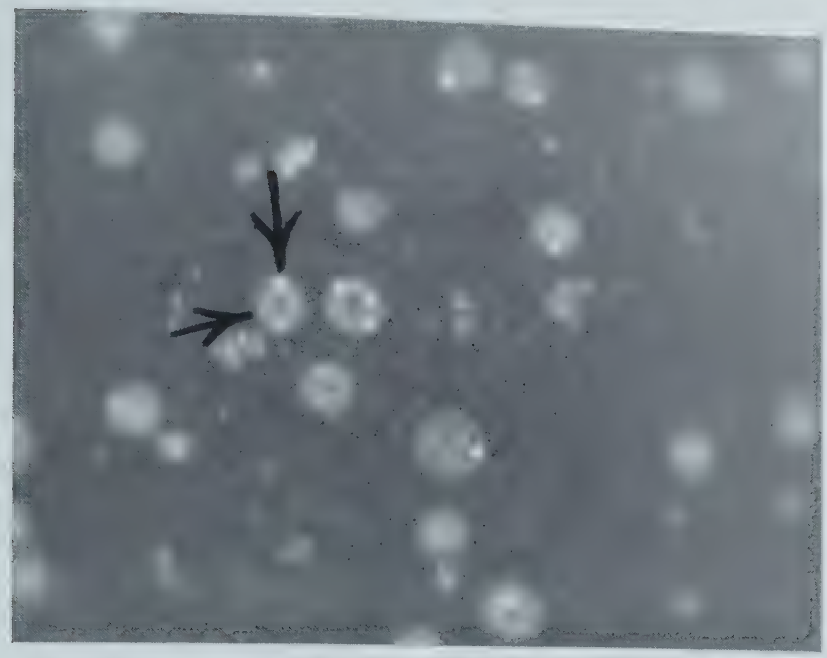


FIG. 2. *Anaplasma marginale* in blood film (from acutely infected cow) stained with fluorescein isothiocyanate-labeled bovine antiglobulin (about × 1000). Note classic marginal body (larger) and initial anaplasma body (smaller). From Ristic and White (1960); photograph obtained through the courtesy of Dr. M. Ristic and published with the permission of the editors of *Science*.

and of a deer shows that the classic marginal inclusion body of *Anaplasma* consists of subunits called initial bodies (Figs. 3a, 3b) (Ristic, 1960; Ristic and Watrach, 1960). Single initial bodies are present in erythrocytes and seem to represent the form which persists in healthy carrier animals. Each initial body contains several dense aggregates of finely granular material. This interesting agent of a disease of great economic importance deserves much more fundamental investigation by modern methods than it has yet received.

Kappa of *Paramecium* is a rod-shaped structure varying in length from 0.6 to as much as 10μ. When examined in the living state with phase contrast microscopy it strikingly resembles bacteria (Preer and Stark, 1953). Double forms suggesting binary fission are often seen (Fig. 4). It differs from bacteria in that no nuclear structure is demonstrable; the deoxyribonucleic acid seems to be uniformly distributed.

Individuals of *Paramecium aurelia* of appropriate genetic constitution, when infected with kappa, produce a poison, paramecin, which is toxic to uninfected individuals (Sonneborn, 1943, 1949). Ordinarily the rate of multiplication of kappa particles keeps pace with that of the host, so that the parasites are transmitted at cell division to the daughter cells. Under certain cultural conditions the multiplication of kappa can be inhibited sufficiently so that the host cells grow away from it and an

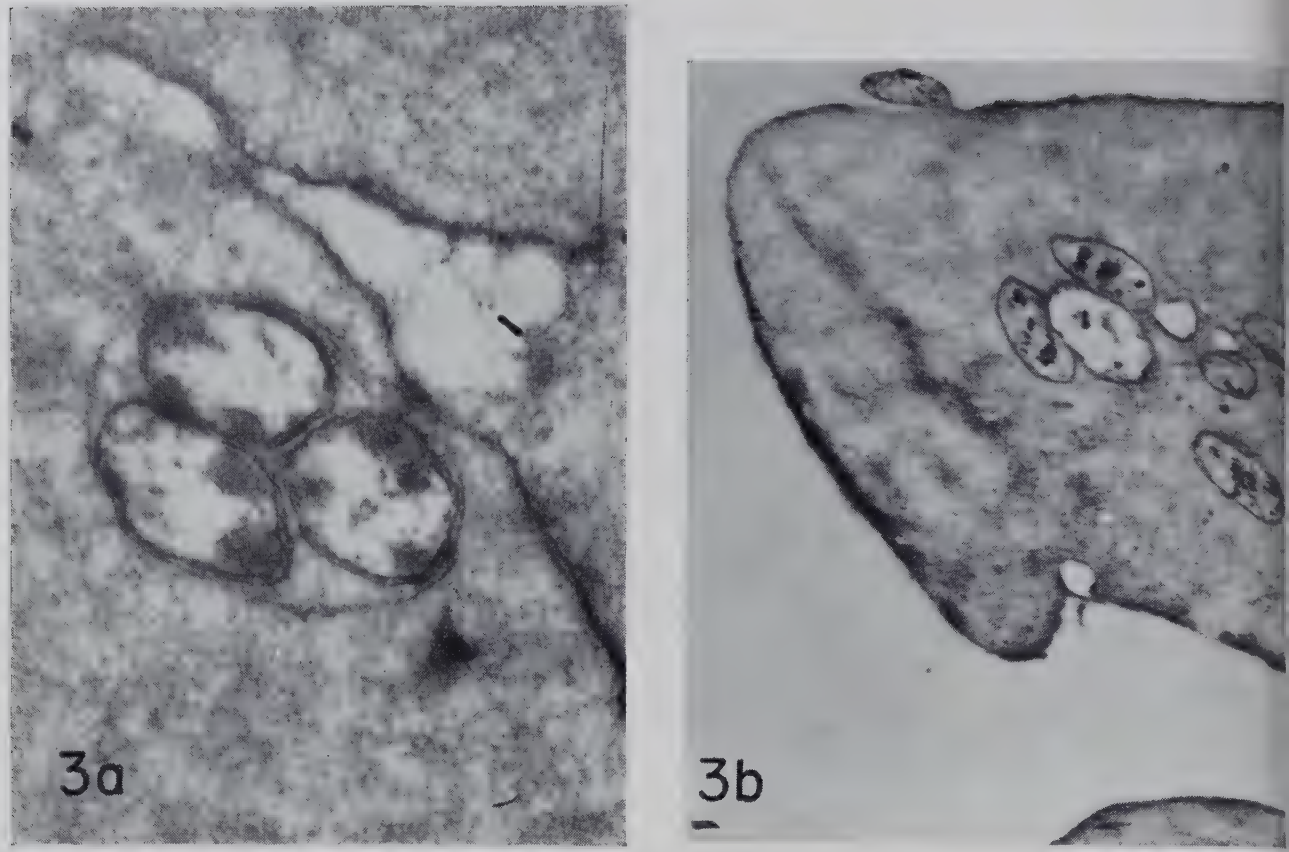


FIG. 3a. Electron micrograph of thin section of erythrocyte of deer with anaplasmosis showing group of 3 initial bodies. Note their double membranes. Each body is 300–400 mμ in diameter. Fixed with 2 % $KMnO_4$. 3b. Thin section of erythrocyte of an infected calf showing several initial anaplasma bodies. Note an initial body at the surface of the red cell and the absence of the red cell membrane at the site of attachment. Fixed in 0.1 % O_sO_4 with 0.3 % $MgCl_2$ 6 H_2O. Magnification: ×30,000. From Ristic (1960). Both electron micrographs kindly supplied by Dr. M. Ristic. Figure 3b published with the permission of the editors of the *Journal of the American Veterinary Association.*

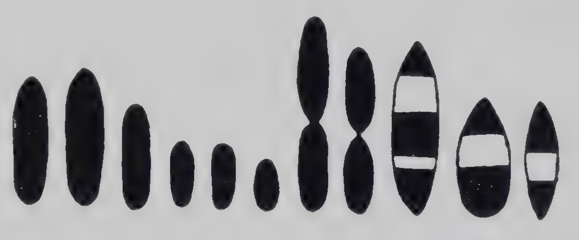


FIG. 4. Different forms of kappa as seen with bright phase. No fixation, paramecia broken. From Preer and Stark (1953); reproduced with the kind permission of the author and Academic Press, Inc.

uninfected strain is obtained. Such a strain can be reinfected by contact with an extract of infected paramecia (Sonneborn, 1949). Although kappa was described originally as a plasmagene, at present it seems difficult to find any sound basis for considering it anything but a peculiar kind of intracellular parasite (Lindegren, 1957).

H. Viruses

Viruses are defined by their very small size (at or below the limits of the light microscope) and their obligate intracellular parasitism. We have already seen that many kinds of microorganisms visible with the light microscope are obligate intracellular parasites. This leaves only size as the criterion. Accordingly, it is not surprising that there are good reasons for believing viruses to be a heterogeneous group (Luria, 1953). Some viruses may represent cytoplasmic or nuclear genes which have become capable of transmission to, and multiplication in, different kinds of cells. Other viruses may represent an extreme of parasitic degeneration. Some of these might be derived from protozoa, others from fungi, others from bacteria or rickettsiae.

Since the viruses are treated in detail in Chapter I, they will not be discussed further in the present chapter except where they illustrate particularly well some general aspect of intracellular parasitism or show a parallelism with other kinds of intracellular parasites.

I. Intranuclear Parasites

Most intracellular parasites develop in the cytoplasm of the host cell, but there are a number which multiply only, or preferably, in the nucleus. The polyhedral viruses of insects appear exclusively in the nuclei of the infected cells, where they are associated with the characteristic polyhedral inclusion bodies (Steinhaus, 1949). The rickettsiae of spotted fever (*Rickettsia rickettsii*) are typically intranuclear in their tick host and often also in tissue culture (Wolbach, 1919; Pinkerton and Hass, 1932). Among the protozoa, the coccidian parasite of cattle *Eimeria alabamensis* is restricted to the nuclei of the intestinal epithelial cells (Fig. 5) (Davis *et al.*, 1957). Even less is known about the conditions which restrict certain parasites to an intranuclear development than about the factors which impel parasites to invade particular types of cells (see below).

III. THE METHODS OF ENTRY INTO THE HOST CELL AND THEIR RELATION TO HOST PARASITE SPECIFICITY

Despite the taxonomic diversity of intracellular parasites, their physiological relationships to their host cells exhibit so much in common that they can be discussed best in functional rather than taxonomic categories. This is not so astonishing when we remember that all parasites have had to solve much the same problems in order to exist. Like extracellular parasites, intracellular ones must have ways of dissemination so that they can reach fresh hosts, and they must have ways of gaining entry into

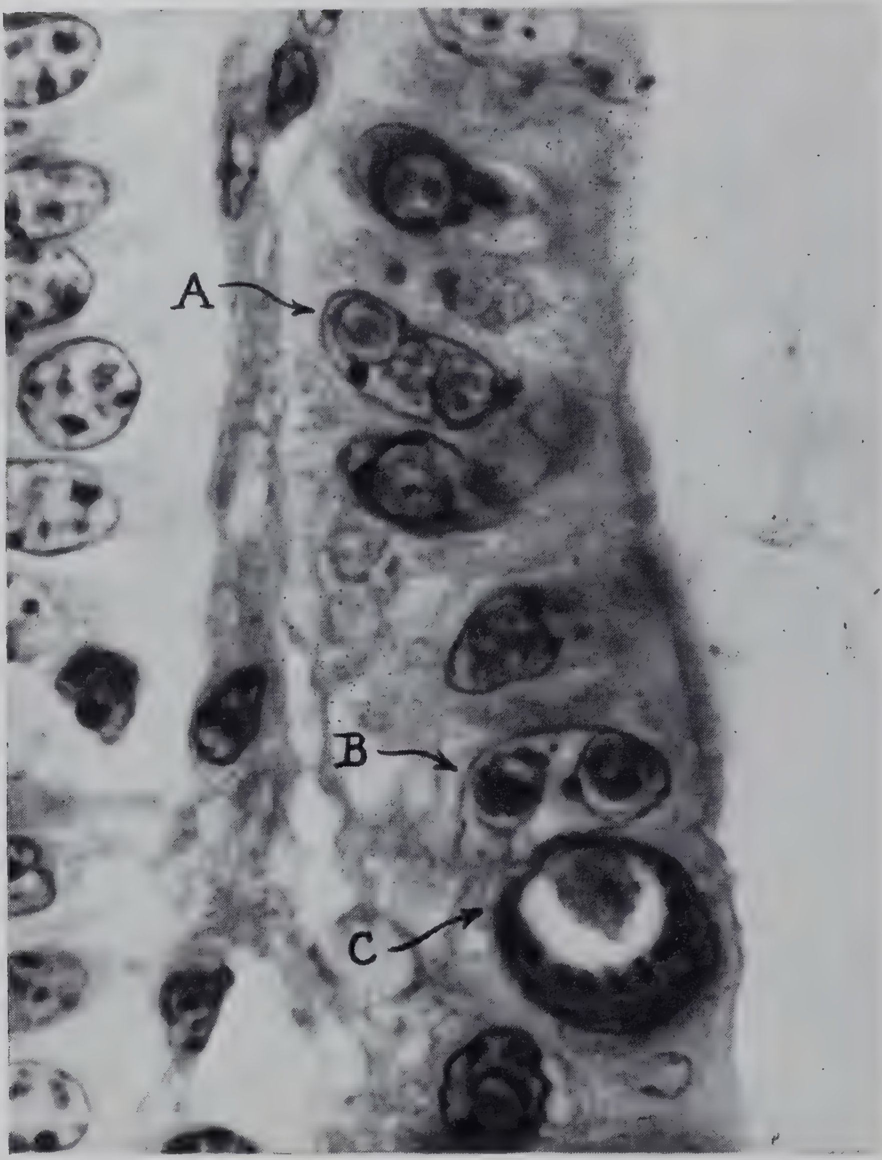
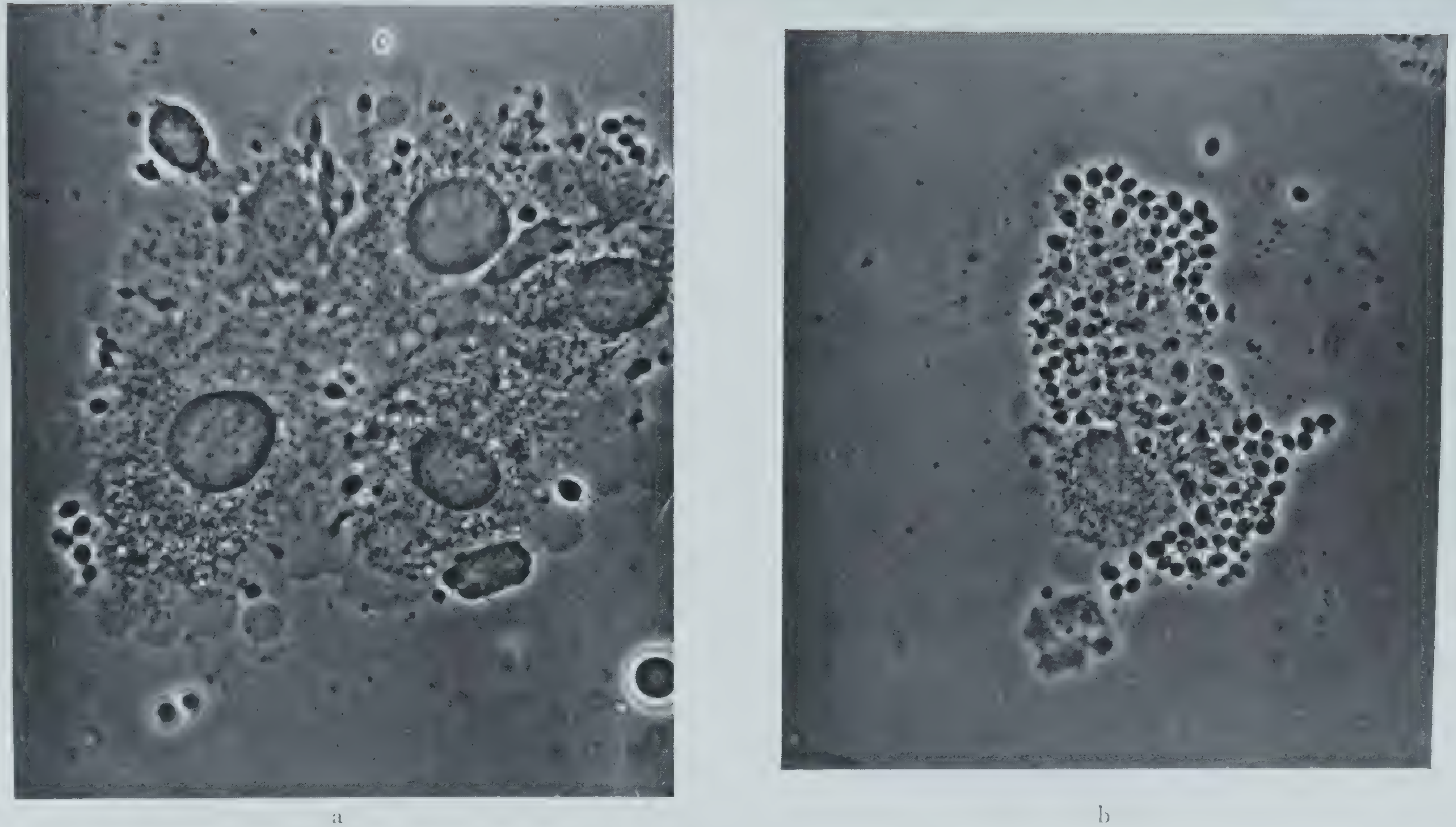


FIG. 5. An intranuclear coccidian parasite of cattle. Developmental stages of *Eimeria alabamensis* in nuclei of intestinal epithelial cells 4 days after infection showing multiple invasion (*A*, *B*) and a schizont (*C*). Magnification: ×1565. From Davis *et al.* (1957); photograph obtained through the courtesy of the authors and published with the permission of the editor of the *Journal of Protozoology*.

the host. In addition, they must be able to penetrate particular host cells and obtain from these the nutrients necessary for growth and reproduction. Finally, they must have means for leaving the host cell. The host cell is the immediate environment of an intracellular parasite, so that a study of the parasite's ecology becomes essentially a study in cell physiology.

A. *Passive Entry*

Parasites may gain entry to a host cell in ways involving no activity of their own. Viruses of plants are often injected directly into phloem



a

b

Fig. 6. Living cells from the spleen of a hamster infected with *Leishmania donovani*. Phase contrast; magnification: ×776. *a*. Lightly infected cells with parasites only at the periphery. *b*. A heavily infected cell. (Original.)

cells by the stylets of the aphid or leafhopper which serves as a vector (Day and Bennetts, 1954).

Many parasites are taken up by the activities of the host cell, either by phagocytosis or by pinocytosis (see Chapter 3). The latter may be particularly important for some of the smaller parasites, such as rickettsiae, and for host cells having no phagocytic activity. Tubercle and leprosy bacilli (Shepard, 1955) and *Leishmania donovani* are presumed to be phagocytosed by their host cells. Figure 6, a shows recently infected cells from the spleen of a hamster with *L. donovani*. Note that the few Leishman-Donovan bodies (leishmanias) are at the periphery of the phagocytic host cells. Cells at a later stage of infection are completely filled with parasites (Fig. 6, b). No highly specific mechanisms are here involved. The parasites are taken up much as would be any other particle of similar size. The specificity begins with the fact that, whereas dead organic material or nonparasitic organisms would be digested by the phagocytic cell, the intracellular parasites resist digestion and indeed multiply within the cell. An interesting example is provided by the behavior of the facultative intracellular parasite *Brucella* in tissue cultures of chick embryo fibroblasts (Holland and Pickett, 1956). Smooth *Brucella* variants were ingested by the cells and multiplied extensively within them. Nonsmooth variants were also ingested, but they neither multiplied nor survived long intracellularly.

B. Active Entry

Specificity appears at the outset, however, when the host cell is entered in a manner demanding some activity on the part of the parasite.

1. Rust Fungi

Spores of rust fungi will germinate under apppropriate conditions of temperature and moisture. If the spore is on a suitable species of plant, the germ tube will then penetrate either directly through the cuticle (if the fungus is in the monokaryotic stage) or through stomata (if it is dikaryotic) and form a very small intercellular mycelium. At the tip of a hypha in contact with the outer wall of a host cell a short terminal cell is formed. This haustorium mother cell becomes closely appressed to the host cell wall, which it then penetrates through a fine pore. The contents of the fungus pass into the host cell, forming the haustorium (Walker, 1950). The successful completion of this process of penetration depends on the precise genetic constitution of both the parasite and the host. Certain varieties of wheat, for example, are resistant to infection by particular races of *Puccinia graminis*. The resistance may be a morphologic one depending on the formation of a relatively large amount of thick-walled collenchyma. More often, however, it depends on a protoplasmic incompatibility between host and parasite. The fungus invades the intercellular spaces in the usual way and even forms a small haustorium, but the haustorium collapses and the host cell dies, resulting in the death of the parasite. It would be nice to know the biochemical basis of this reaction.

2. *Microsporidia*

The resistant spores of the Microsporidia contain a special coiled structure, the polar filament. When the spores are ingested by an appropriate host, or also under certain conditions *in vitro* (Ohshima, 1927; Trager, 1937; Dissanaike and Canning, 1957), a rapid extrusion of the polar filament occurs. Attached to the distal tip of the long extruded filament is the minute uninucleate amebula which initiates infection in the new host. It seems likely that the rapid extrusion of the polar filament helps to bring the amebula into contact with host tissue and may aid in penetration of the host cell.

3. *Malaria Parasites*

The erythrocytic stages of malarial parasites develop in cells which certainly have no phagocytic activity. It must be assumed that the infective forms, the merozoites, penetrate the surface membrane of the erythrocyte by means of mechanical and enzymatic activities of their own. In animals infected with malaria, sporulation is often a highly synchronous process (accounting for the periodicity of the chills and fever in man). Yet even in very heavy infections when large numbers of merozoites are breaking out of the red cells in which they have been formed and are invading new red cells, an extracellular parasite can virtually never be seen. The liberated merozoites must either rapidly enter new red cells or be taken up and destroyed by phagocytes of the host. Although the liberation of merozoites has been observed repeatedly in preparations of infected blood under the microscope, their penetration into a new erythrocyte has been reportedly seen only twice (Schaudinn, 1902; Trager, 1956). In the more recent observation, one freshly liberated merozoite was seen to come in contact at its anterior, pointed end with the surface of an uninfected red cell, which it penetrated in less than half a minute.

Huff *et al.* (1960) have recently observed the penetration of chick embryo cells in tissue culture by the exoerythrocytic forms of the malarial parasites *Plasmodium gallinaceum* and *P. fallax*. These forms develop within reticuloendothelial cells (Huff and Coulston, 1944) and, like the erythrocytic forms, reproduce by a process of schizogony (Fig. 7). Mero-

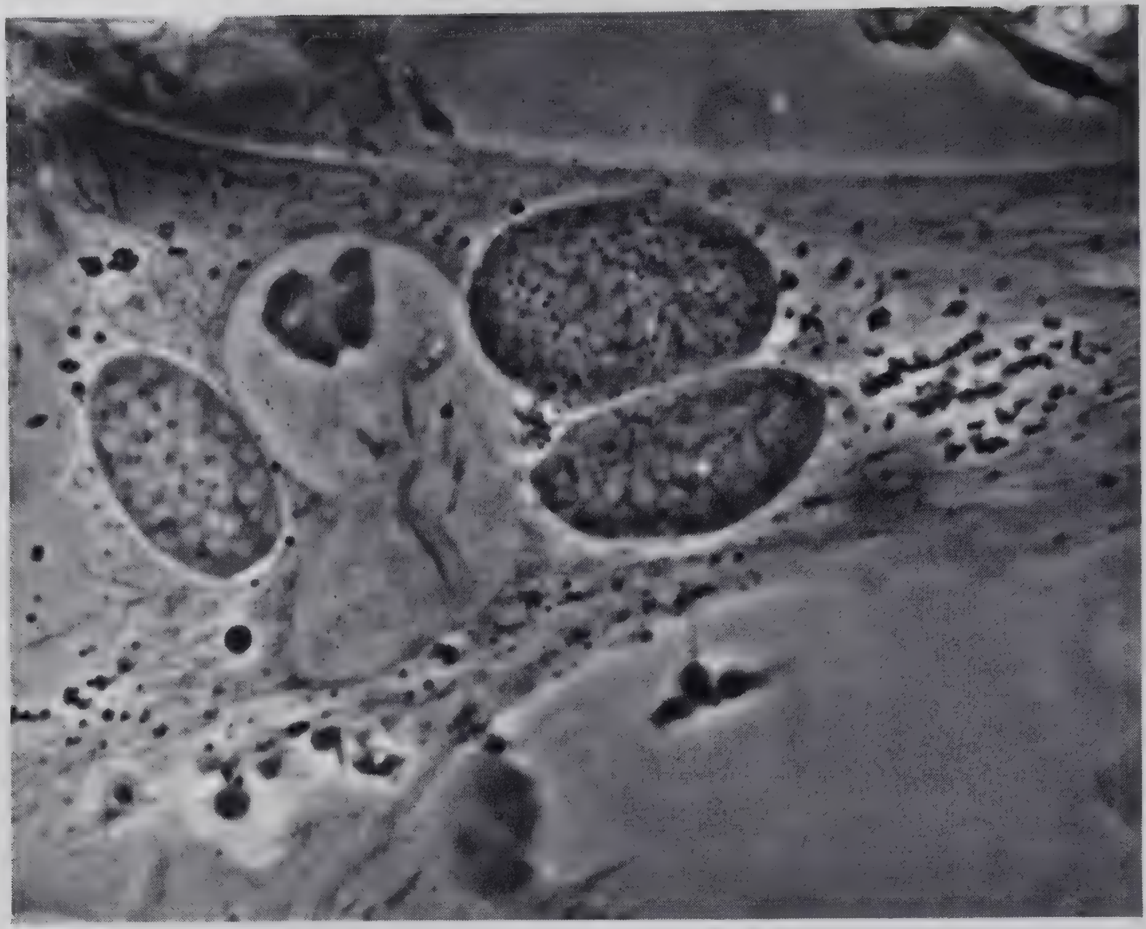


FIG. 7. *Plasmodium fallox.* Exoerythrocytic stages in cell from tissue culture of liver of infected chick embryo. Three schizonts may be seen, one on one side and two on the other side of the host cell nucleus. Magnification: ×700. (Photograph obtained through the courtesy of Dr. C. G. Huff, Naval Medical Research Institute.)

zoites liberated in cultures of infected tissue sometimes put out a very fine elongate filament visible with phase contrast microscopy. In a few instances the filament was seen to make contact with a host cell and to enter the cell by a corkscrewlike motion. Perhaps this situation is analogous to acrosome filament formation by the sperm of some marine invertebrates (Dan, 1954; Colwin and Colwin, 1957) when they enter an egg cell—an example of a different kind of specific penetration of one cell by another.

The erythrocytic stages of malaria are remarkably host-specific. The specificity must in most instances be a resultant of a number of different factors including serological and other extracellular conditions in the host, the readiness with which the host's red blood cells are attacked by the parasite, and the suitability of the red cells for development of the parasite. Even within the same host animal, different species of malaria show preferences for different types of red cells. In man *P. vivax* preferentially invades reticulocytes (Ferrebee *et al.*, 1946). *P. berghei* in the rat shows an extremely high selectivity for young red cells (but not too young) (Singer, 1954; Zuckerman, 1957). There is good evidence that people heterozygous for the sickle-cell trait are relatively more resistant to *P. falciparum* malaria, thereby accounting most probably for the high prevalence of this trait in malarious countries (Allison, 1957). Perhaps this is because these parasites selectively avoid cells containing hemoglobin S, a not unreasonable assumption in view especially of the fact that hemoglobin forms an integral part of the red cell membrane (Moskowitz and Calvin, 1952).

An interesting beginning toward studying some of these problems has been made by McGhee (1950). He found that chick embryos infected with the bird-malaria parasite *P. lophurae* could be injected intravenously with washed suspensions of erythrocytes of different species of animals. Four hours later the degree of penetration of these introduced red cells (which differed morphologically from those of the chick) could be determined quantitatively. It turned out (McGhee, 1953b) that duck erythrocytes were so much more susceptible than chick embryo erythrocytes that all of the merozoites which succeeded in entering cells entered duck cells, despite the fact that duck cells were only about one-third as numerous as chick cells. The presence of two or more parasites in a duck red cell tended to discourage penetration by additional parasites, a fact reminiscent of the exclusion phenomenon so well shown for bacteriophage and more recently for animal viruses as well (Dulbecco, 1957). More interesting yet, the erythrocytes of certain species of mammals, notably the mouse, were invaded by this avian parasite. Erythrocytes with a high sodium content (dog, sheep, cattle) were not parasitized by *P. lophurae* (McGhee, 1950). It may be mentioned in this connection that parasites of the genus *Plasmodium* have been found so far only in animals whose erythrocytes are high in potassium (reptiles, birds, rodents, primates), whereas parasites of the genus *Babesia* occur mainly in animals having erythrocytes with a high sodium content (ruminants, dogs), but also in rodents.

When introduced into infected chick embryos, the red cells of weaned or older rats were completely immune to *P. lophurae*, whereas the red cells of newborn rats were quite susceptible (Table I) (McGhee, 1953a). If we knew the biochemical basis for this difference, we would have made significant progress toward understanding host-parasite specificity at the cellular level. Perhaps fetal hemoglobin of the rat differs from adult hemoglobin and is more attractive to the parasites. The reverse situation has been suggested (Allison, 1957) as an explanation of the relatively

TABLE I

THE COMPARATIVE SUSCEPTIBILITY OF THE ERYTHROCYTES FROM RATS OF VARIOUS AGES, WHEN INTRODUCED INTO CHICK EMBRYOS INFECTED WITH *Plasmodium lophurae*[a]

Age, in days, of donor animal	Time, in hours, after foreign cell introduction					
	0			4		24
	Parasites per 10,000 chick erythrocytes	Merozoites per 10,000 chick cells	Number rat cells per 10,000 total cells	Per cent merozoites in rat erythrocytes	Parasites per 10,000 rat erythrocytes	Parasites per 10,000 rat erythrocytes
1	2100	3900	2800	3.00	98	1040
7	1125	1600	3200	3.00	19	170
13	1973	2100	3400	1.40	39	120
17	1000	2500	2900	0.40	8	52
21	2700	3100	2800	0.20	7	
30	2500	3800	3700	0.10	5	
40	1800	882	2600	0.04	0.4	1

[a] The influence of age of the animal upon the susceptibility of mammalian erythrocytes to infection by the avian malaria parasite *Plasmodium lophurae* (McGhee, 1953a).

high resistance of human infants to malaria during the first three months of life, a period when many of the red cells contain fetal hemoglobin.

The interaction between malarial merozoites and the erythrocyte surface may be at first a physical one, followed then by enzymatic activity (and perhaps also mechanical activity) on the part of the parasite.

4. Bacteriophage; Influenza Virus

A somewhat similar sequence of events has been demonstrated in the invasion of bacteria by bacterial viruses. There is an initial reversible attachment of the phage particles to appropriate host cells. This depends on the nature of the phage and the host and on conditions in the culture medium, and has properties to be expected from the operation of electrostatic forces (Garen and Puck, 1951). Attachment is followed by an irreversible enzymatic transformation which increases the permeability of the host cell wall (Puck and Lee, 1954) and is accompanied by the penetration of the phage deoxyribonucleic acid (DNA) (but not the protein membrane of the phage) into the host cell (Hershey and Chase, 1952). In keeping with this mechanism, it has been found that isolated cell wall material of *Escherichia coli* was converted to nonsedimentable form by interaction with the T phages (Barrington and Kozloff, 1956). The enzyme responsible for this was present in the protein portion of the virus, since virus freed from its DNA by osmotic shock was as active in this respect as intact virus. The specificity of this second reaction seems to depend on the antigenic glycolipoproteins present in the bacterial cell wall Jesaitis and Goebel, 1953). The tail protein is apparently contractile and has phosphatase activity (Dukes and Kozloff, 1959; Kozloff and Lute, 1959).

A not too different situation probably obtains in the infection of host cells by viruses of the influenza group. The interaction between these viruses and certain kinds of red blood cells, although it does not lead to infection of the latter, does provide a partial model for the events which occur when susceptible host cells are penetrated (Hirst, 1942, 1943). Influenza virus first attaches at receptor sites on the surface of the erythrocyte. The attached virus then, by means of an enzyme (Hirst, 1942), initiates a spreading disturbance over the surface of the erythrocyte which destroys receptor sites (Sagik *et al.*, 1954) and also increases the permeability of the red cell. The substrates of the enzymes are mucoproteins (Howe, 1951). The enzymes are glycosidases which liberate neuraminic acid or a derivative of it from glycosidic linkage in the mucoprotein (Gottschalk, 1957; Howe *et al.*, 1957). These viral enzymes may be concerned with escape from, as well as with penetration of, the host cell.

IV. The Intracellular Habitat

A. Physical Characteristics

When a parasite lives inside of a living cell it is sheltered in various physical and mechnical ways from deleterious influences which it might encounter outside of the cell. Since phagocytic cells do not ordinarily ingest other cells of the same organism, parasites lying within such other cells are protected from phagocytosis. This protection breaks down when, as in acute malarial infections, autoantibodies appear and large numbers of uninfected as well as infected erythrocytes are destroyed (Taliaferro and Cannon, 1936). The semipermeable membrane of the host cell protects parasites within the cell from agents such as antibiotics. Thus intracellular tubercle bacilli are not affected by concentrations of streptomycin in the external medium which are lethal for extracellular bacilli (Mackeness and Smith, 1953). Many viruses have been shown not to be affected by specific immune serum once they are within their host cells. The cells themselves of immune animals, however, may in some instances acquire the ability to inhibit the growth of intracellular parasites, an ability not directly related to serological antibodies. *Brucella abortus* multiplied to a much lesser extent in monocytes from immune guinea pigs than in monocytes from normal guinea pigs (Pomales-Lebron and Stinebring, 1957). Similar results have been observed with tubercle bacilli. The nonspecific nature of this reaction has recently been demonstrated. Monocytes from animals immunized to either tubercle bacilli or brucella showed resistance to both (Elberg *et al.*, 1957).

The physical nature of the host cell may also provide the parasite with particular, essential conditions of osmotic pressure, viscosity, and surface structure. Very little is known about these aspects of cell-parasite relationships. The higher osmotic pressure which evidently exists at least in some kinds of cells (Opie, 1956) may be required for the growth of some parasites. Surface structure may be more important than one might at first suppose. An example of the importance of surface structures on a relatively macroscopic scale is provided by certain free-living marine ciliates. These occur only in the tidal zone of such beaches as have sand grains of a particular average size. Whether the beach is on Cape Cod or on the coast of France matters little: if the sand grains are of the right size the ciliates are present (Fauré-Fremiet, 1951).

B. Biochemical Characteristics

The nutritive and other biochemical factors of the host cell undoubtedly are of overshadowing importance to the parasite. An intracellular parasite must be able to obtain either directly from its host cell, or from the external medium by way of the host cell, all its essential nutrients. Since most intracellular parasites have not been grown apart from living host cells, the study of their nutrition and metabolism is fraught with difficulty. This study has been approached in three ways: (1) study of the metabolism of the host cell-parasite complex; (2) short-term metabolic studies of surviving parasites freshly removed from their host cells; (3) longer-term studies of parasites removed from their host cells, involving attempts to substitute a nonliving culture medium for the host cell.

1. *Studies with Parasite-Host Cell Complex*

The results obtained by the first method are not easily interpreted. Even the delicate tracer techniques fail to answer unequivocally the question whether substances from the external medium used by a parasite are utilized directly or are first incorporated into some component of the host cell. By means of P^{32} it has been found that phage phosphorus is derived both from the culture medium and from phosphorus compounds present in the bacterial cell before infection, notably the constituents of the bacterial DNA (Barry *et al.*, 1950; Kozloff and Putnam, 1950; Labaw, 1951; Weed and Cohen, 1951; Putnam *et al.*, 1952; Siddiqi *et al.*, 1952; Kozloff, 1953). The proportion of phosphorus derived from these two sources varies greatly with the particular bacteriophage. For the T7 phage, 60–90% was obtained from the host DNA, whereas for T6 phage only about 30% was so derived, the remainder coming from the medium. The nitrogen of the viral DNA similarly may be derived from host DNA to a greater or lesser extent. In general, the nitrogen of the viral protein originates largely from compounds assimilated from the medium after infection, though here again there is considerable variation and in T7, 40% of the protein nitrogen originates from bacterial material assimilated before infection (Putnam *et al.*, 1952).

Malarial parasites likewise incorporate P^{32} from the medium bathing their host red cells into their own DNA (Table II) (Clarke, 1952a; Whitfeld, 1953). At least one species (*Plasmodium gallinaceum*), like some of the bacteriophages, also makes use of the nucleic acid of the host erythrocyte (Lewert, 1952a, b). Nitrogenous substances likewise come from both sources. *P. knowlesi* derives 80% of its methionine and cystine from the host erythrocyte, only 20% from external sources (Fulton and Grant, 1956).

The nutritional requirements of the host cell-parasite complex have been studied in intact host organisms as well as in isolated host cell systems. Becker and Smith (1942) early observed that the intracellular coccidian parasite *Eimeria nieschulzi* developed better in rats on an

TABLE II

P³² ACTIVITY OF FRACTIONS OF NORMAL ERYTHROCYTES AND ERYTHROCYTES PARASITIZED BY *Plasmodium gallinaceum* AFTER EXPOSURE FOR 22 HOURS *in vivo* or *in vitro*[a]

Erythrocytes		P³² activity of cell fraction, counts/min./ml. cells		
		Acid-soluble	Alcohol-ether-soluble	Nucleic acid
In vivo	Normal	640	210	30
	Parasitized	2100	2230	1700
In vitro	Normal	440	60	40
	Parasitized	860	340	730

[a] The use of phosphorus 32 in studies on *Plasmodium gallinaceum*. I. The development of a method for the quantitative determination of parasite growth and development *in vitro* (Clarke, 1952a).

adequate diet than in rats fed a diet deficient in pantothenate. Soon thereafter it was found that a malarial parasite (*Plasmodium lophurae*) survived longer in erythrocyte suspensions maintained *in vitro* in the presence of added pantothenate (Trager, 1943). Another species of avian malaria, *P. gallinaceum*, was shown not to develop in chickens deficient in pantothenate or fed analogs of pantothenic acid (Brackett *et al.*, 1946). Indeed, such analogs of pantothenate were effective antimalarial drugs (Marshall, 1946). Pantothenic acid is certainly a nutritional factor which must be supplied to the malaria parasite-host erythrocyte complex in order for the parasite to develop. *p*-Aminobenzoic acid is another such factor. This was first shown for *P. knowlesi* maintained intracellularly in suspensions of host monkey red cells (Ball *et al.*, 1945). More recently, *p*-aminobenzoic acid has been found essential in the host's diet for the development of *P. berghei* in mice and rats (Hawking, 1954). By similar means evidence has been obtained that the malaria parasite-host erythrocyte complex requires for the development of the parasite ascorbic acid (McKee and Geiman, 1946), thiamin (Rama Rao and Sirsi, 1956), riboflavine (Seeler and Ott, 1946), biotin (Trager, 1947b), and methionine (Taylor, 1956).

It is interesting to examine the parallel results obtained with virus-host cell systems. When T2 bacteriophage developed in *Escherichia coli* maintained in a simple synthetic medium of salts, lactate and ammonium, the latent period was much longer and the burst size much smaller than in a broth medium. The addition to the simple medium of valine, isoleucine, leucine, phenylalanine, histidine, arginine, lysine, aspartic acid, glutamic acid, methionine, trytophan, tyrosine, and adenine gave phage development with a latent period only 1–2 minutes longer and a burst size within 10% of those in broth (Fowler and Cohen, 1948; Cohen and Fowler, 1948; Cohen, 1949).

The growth of a number of animal viruses in tissue culture or in an intact host is inhibited by nutritional deficiencies or by antimetabolites in the culture medium or in the diet of the host. Both glucose and glutamine were essential for the formation of poliomyelitis virus in HeLa cells in culture (Eagle and Habel, 1956) and glucose was similarly a requisite for influenza virus formation (Daniels *et al.*, 1952). Strain WS of influenza virus could grow with pyruvate in place of glucose but another strain (NWS) could not (Levine *et al.*, 1956). Influenza virus was inhibited by canavanine, an inhibition which could be reversed by L-arginine (Pilcher *et al.*, 1955). Oxythiamine and deoxypyridoxine inhibited both mumps and influenza virus in tissue cultures without any apparent toxic effects on the cultures themselves (Cushing and Morgan, 1952). Other antimetabolites inhibiting viral synthesis were: for influenza, benzimidazole derivatives (Tamm and Tyrrell, 1954), β-2-thionylalanine, all of which also markedly inhibited respiration of host cells (Gifford *et al.*, 1954); for Russian spring-summer encephalitis, 2, 6-diaminopurine (Friend, 1951), a compound which also inhibits kappa (Stock *et al.*, 1951); for mouse encephalomyelitis, methoxinine (Pearson *et al.*, 1955). The cytopathogenic action of poliomyelitis virus on monkey kidney cells was much delayed when cystine was omitted from the maintenance medium (Dubes, 1956). In order to get good growth of psittacosis virus in tissue culture, the medium had to contain various water-soluble vitamins and amino acids, notably phenylalanine and tryptophan (Weiss, 1955; Johnson and Morgan, 1956; Heggie and Morgan, 1956). Psittacosis virus, like others of its group, and like malaria parasites, is inhibited by sulfadiazine, and the inhibition is counteracted in a competitive manner by *p*-aminobenzoic acid and in a noncompetitive manner by pteroylglutamic acid (Morgan, 1948).

Must we conclude from results of this kind that influenza virus utilizes thiamin, or that psittacosis virus and malaria parasites utilize *p*-aminobenzoic acid to synthesize pteroylglutamic acid? As long as the parasite is developing within its host cell there is no way of knowing whether the required metabolite is used directly by the parasite or whether it is used by the host cell to synthesize some other material which in turn is essential to the development of the parasite. A case in point has been provided by a study of an avian malaria parasite (*Plasmodium lophurae*). We have already noted that this organism appears to require pantothenate

TABLE III

THE EFFECT OF TWO PREPARATIONS OF COENZYME A IN THE PRESENCE AND ABSENCE OF ADDED PANTOTHENATE ON THE EXTRACELLULAR SURVIVAL OF *Plasmodium lophurae* AS SEEN IN STAINED FILMS AFTER 3 DAYS *in vitro*[a, b]

		Pantothenate (μg. per ml.)		Coenzyme A			No. of degenerate parasites
					Units per ml.		
Expt.	Flasks	Added	Total[c]	Preparation	Added	Total[c]	in 200 counted
A	1, 2	0	—		8	—	38, 24
	3, 4	0	—	Armour concentrate	8	—	13, 15
	5, 6	4.9	—		0	—	27, 16
	7, 8	4.9	—	Armour concentrate	8	—	20, 9
B	1, 2	0	—		0	—	21, 36
	3, 4	0	—	Armour concentrate	8	—	9, 19
	5, 6	4.9	—		0	—	38, 34
	7, 8	4.9	—	Armour concentrate	8	—	35, 11
C	1, 2	0	0.4		0	0.5	14, 39
	3, 4	0	0.6	Armour concentrate	8	9	27, 13
	5, 6	0	0.6	Pabst	8	9	18, 7
D	1, 2	0	0.4		0	0	15, 15
	3, 4	0	0.4	Pabst	8	8.5	6, 9
	5, 6	4.9	5.0		0	1	27, 24
	8	4.9	5.0	Pabst	8	8.5	6

[a] The culture medium was duck erythrocyte extract prepared and supplemented in the manner previously described. Fresh medium and supplements of adenosine triphosphate, sodium pyruvate, and coenzyme A were added after the first 18 hours and at 12-hour intervals thereafter.

[b] Coenzyme A and the malaria parasite *Plasmodium lophurae* (Trager, 1954).

[c] Determined by microbiological assay.

when it is developing within host erythrocytes maintained *in vitro*. When the organisms were removed from their host cells, however, and maintained extracellularly in a medium based on erythrocyte extract, it developed that their survival was favoured by the addition of coenzyme A to the medium (Trager, 1954). Pantothenate had no effect (Table III). Furthermore it was found that the erythrocytes of birds infected with *P. lophurae* had a higher coenzyme A content than those of uninfected birds, the increased coenzyme A being contained in the parasites themselves. The results fit the hypothesis that the parasites satisfy their requirement for an external source of preformed coenzyme A by taking it from the host red cell, which in turn requires an external source of pantothenate to enable it to manufacture the coenzyme A.

Just as the results of nutritional experiments with the host cell-parasite complex are difficult to interpret, so too are the results of measurements of the metabolism of the complex. If, as is very often the case, the complex has a higher metabolic activity than the uninfected host cell, how much of this represents the metabolism of the parasite? We cannot arrive at a reasonable answer merely by subtracting the activity of the uninfected cell from that of the cell-parasite complex. The infection of a cell by a parasite often leads to grossly visible morphological changes, and one must assume that it always leads to physiological changes. An infected cell is an altered cell, and there is no justification for considering its metabolism to be at all similar quantitatively or even qualitatively to that of a corresponding uninfected cell.

A large change in metabolism can sometimes be brought about by the introduction of a single cofactor. For example, mature human erythrocytes show an extremely low level of glucose oxidation which, however, is stimulated twenty- to fiftyfold in the presence of catalytic amounts of methylene blue or other dyes, or purified diaphorase (Huennekens, 1957). Hence these cells are not deficient in the enzymes requisite for glucose oxidation, but rather in a cofactor. It is easy to see that infection of such a cell by a malarial merozoite, for instance, might introduce a cofactor with a resulting high respiratory activity of the infected cell. Hence the higher oxidative metabolism of erythrocytes infected with malaria parasites, which has been observed repeatedly (McKee, 1951), may be only partly a result of activity of the parasites themselves.

2. *Studies with Parasites Removed from the Host Cell*

Because of these difficulties in interpretation of experiments with the host cell-parasite complex (however interesting and important the results are), attempts have been made to study the metabolism of intracellular parasites removed from their host cells. Only a relatively few kinds of parasites have been studied in this way. In general viruses, even the larger ones, have not been found to have any metabolic activity when freed from their host cells (Gottschalk, 1957), and it has been concluded that they lack oxidative enzyme systems. For the larger viruses especially, this conclusion must be treated with some caution (see also Weiss, 1955). Rickettsiae likewise appeared to have no independent respiratory activity until an appropriate substrate was discovered (Bovarnick and Snyder, 1949). The fact that copper and flavine-adenine dinucleotide are both integral parts of the vaccinia virus particle suggests the presence of some oxidative mechanism, even though none of the enzymes ordinarily associated with these cofactors has yet been demonstrated in the virus (Gottschalk, 1957). Recently cytochrome c reductase activity has been found in the large meningopneumonitis virus (Allen and Bovarnick, 1957). Aside from the chiefly negative results with viruses, most of the work has been with leprosy bacilli, rickettsiae, and malaria parasites and can conveniently be treated in these three categories.

a. Murine leprosy bacilli free from host tissues have an endogenous respiratory activity (QO_2 of 20–70 µl. O_2 per hour per milligram nitrogen) which resembles that of other mycobacteria in being sustained by small amounts of CO_2 and by adequately buffered solutions at pH 7.5 (Gray, 1952). They differ markedly, however, from the cultivable pathogenic mycobacteria in their inability to utilize substrate *in vitro* (though purified bovine serum albumin has a favorable effect) and in their susceptibility to natural inhibitors in rat serum (Table IV) (Hanks, 1954a). It is of the greatest interest that tuberculosis organisms freshly prepared from infected tissue had a quite different metabolism from that of the same strain grown in culture—the former had much less ability to utilize substrate added *in vitro* (Segal and Bloch, 1956). No true culture of either human or murine leprosy bacilli has yet been obtained. Indeed, upon incubation of a suspension of *Mycobacterium lepraemurium* at 37° C. under relatively favorable conditions, both the respiratory activity and infectivity were 60% gone after 24 hours (Hanks, 1954c).

TABLE IV

FAVORABLE INFLUENCE OF PURIFIED BOVINE SERUM ALBUMIN AND ADVERSE EFFECT OF RAT SERUM ON THE HYDROGEN TRANSFER CAPACITY OF LOW-QUALITY MURINE LEPROSY BACILLI[a]

Protein concentration	Hydrogen transfer capacity (%)
5% Bovine serum albumin	550
1% Bovine serum albumin	150
0% (Intracellular balanced salt solution)	100
1% (Rat serum 14.5%)	75
5% (Rat serum 71.5%)	50

[a] Modified from Hanks (1954b). Influence of physical and chemical factors on the hydrogen transfer capacity of murine leprosy bacilli.

b. Rickettsiae. Oxygen consumption by suspensions of isolated rickettsiae was first demonstrated for typhus rickettsiae in a medium of high potassium content with casein hydrolyzate as the substrate (Bovarnick and Sydner, 1949). The casein hydrolyzate could be replaced completely by glutamic acid. There was no utilization of glucose even in the presence of added adenosine triphosphate, cozymase, hexose diphosphate, Mg^{++}, and Mn^{++}. Rickettsiae oxidizing glutamate formed CO_2, NH_3, and aspartate. Moreover, they could be shown to possess a transaminase which enabled them to take up O_2 rapidly in the presence of a mixture of aspartate and α-ketoglutarate (Bovarnick and Miller, 1950). Pyruvate, α-ketoglutarate, fumarate, malate, oxaloacetate, and succinate were also oxidized by the rickettsiae (though not nearly so effectively as glutamate), indicating that the organisms probably oxidize glutamate via the Krebs citric acid cycle (Wisseman *et al.*, 1951, 1952). A small but highly significant phosphorylation accompanied the oxidation of glutamate by typhus rickettsiae in the presence of adenosine diphosphate, hexokinase, and glucose (Table V). The phosphorylation was prevented both by 10^{-4} *M* KCN, which abolished the O_2 uptake, and by 10–^{4} *M* dinitrophenol, which had no effect on the rate of O_2 uptake. The latter compound as well as the former inhibited the hemolytic effect of rickettsiae, showing that phosphorylation rather than respiration was indispensable for this toxic

TABLE V

UPTAKE OF INORGANIC PHOSPHATE DURING OXIDATION OF GLUTAMATE BY MADRID E STRAIN OF TYPHUS RICKETTSIAE IN PRESENCE OF ADP, HEXOKINASE, AND GLUCOSE[a, b]

Conditions	Oxygen uptake	P_i	G–6–P	P_i	G–6–P
	(μatoms)	(μmoles)	(μmoles)	(μmoles)	(μmoles)
Standard, zero time	—	10.4	0.0	—	—
Standard	13.7	5.53	5.15	–5.19	4.83
CoA omitted	13.0	7.03	3.15	–3.69	2.83
DPN and CoA omitted	7.84	9.6	1.61	–1.12	1.29
KCN, 10^{-4} *M* added	0.0	10.72	0.32	—	—

[a] The reaction time in the experiment was 146 minutes. P_i represents the difference between the inorganic phosphate present in the reaction mixtures containing cyanide at the end of the reaction period and that present in its absence. G–6–P represents the difference between the G–6–P formed in the absence of cyanide and that formed in its presence.

[b] Phosphorylation accompanying the oxidation of glutamate by the Madric E strain of typhus rickettsiae (Bovarnick, 1956).

manifestation of the rickettsiae (Bovarnick, 1956). The addition of diphosphopyridine nucleotide and coenzyme A increased the phosphorylation.

These two coenzymes are of considerable significance to the activity of rickettsiae. Thus, purified preparations of rickettsiae, if held for 18 hours in isotonic salt solution at 0° C., lose their toxicity for mice, their hemolytic activity, their respiratory activity, and their infectivity for chick embryos. From 50 to 100% of the lost activity could be restored by 3 hours' incubation at 33° C. in the presence of diphosphopyridine nucleotide. The extent of restoration was favoured by the further addition of coenzyme A (Bovarnick and Allen, 1957). Interestingly enough, the phenomenon of reactivation of the rickettsiae of Rocky Mountain spotted fever (*R. rickettsii*), which occurs in nature when an overwintered infected tick ingests blood (Parker, 1933), has recently been duplicated in the laboratory with these same coenzymes. When a suspension of avirulent (for guinea pigs) rickettsiae prepared from ticks was incubated for 6 hours at 25° C. with either diphosphopyridine nucleotide or coenzyme A it was converted to the virulent form (Gilford and Price, 1955).

Rickettsiae can be grown readily in tissue culture. Some species can multiply perhaps extracellularly (though this is by no means certain) in the hemolymph of the mealworm *Tenebrio molitor* and the tick *Ornithodoros moubata*, and all can multiply in the hemolymph of the body louse. In lice they can be passed in series by intracoelomic inoculation (Weyer, 1954). These last observations suggest the possibility of extracellular cultivation.

c. *Malaria parasites* removed from their host erythrocytes by hemolysis of the latter with saponin or with a hemolytic antiserum-complement system showed, when first prepared, an O_2 uptake about 50 to 70% that of the intact infected cells (Christophers and Fulton, 1939; Bovarnick *et al.*, 1946a, b; Speck *et al.*, 1946). The rate of O_2 uptake of such extracellular parasites, unlike that of intact parasitized cells, was increased by a number of cofactors including succinate, malate, fumarate, adenylate (or adenosine triphosphate), Mn^{++}, di- or triphosphopyridine nucleotide, thiamin and diphosphothiamin (Table VI) (Speck *et al.*, 1946; Evans, 1946). The parasites were shown to convert glucose to lactate by a process of phosphorylative glycolysis and to oxidize the lactate via the Krebs citric acid cycle, in the same way as vertebrate tissues (Moulder, 1948). The parasites appear to be fully equipped with enzymatic mechanisms for the metabolism of glucose and some other carbohydrates. They also can digest hemoglobin, as is apparent from the formation of hematin (Deegan and Maegraith, 1956) in infected erythrocytes. Theoretically, 70 to 80% of host cell hemoglobin is converted to parasite pigment by *Plasmodium knowlesi* of the monkey (Morrison and Jeskey, 1948; Black, 1947; Ball

TABLE VI

EFFECT OF ACIDS OF TRICARBOXYLIC ACID CYCLE ON RESPIRATION OF PARASITE PREPARATIONS (*Plasmodium gallinaceum*)[a, b]

Substrate added	Increase in oxygen consumption over level without added substrates	
	Parasitized erythrocytes	Free parasites
	(%)	(%)
Pyruvate	111	167
Oxalacetate	51	102
cis-Aconitate	0	44
Citrate	0	16
α-Ketoglutarate	8	88
Succinate	18	96
Fumarate	16	179
Malate	1	204

[a] The samples contained 0.6 cc. of parasitized erythrocytes or 1.2 cc. of free parasites (from different samples of blood) and the substances indicated below in 0.01 *M* concentration, in a volume of 3.0 cc. 0.01 *M* $MgSO_4$, was added to all samples containing *cis*-aconitate or citrate. The samples with free parasites all contained the cofactor mixture and also 0.00033 *M* malate when the substrate was pyruvate. Gas-phase, air; temperature, 40° C.; time, 60 minutes. The values for parasitized erythrocytes are from a single typical experiment; those for free parasites represent the average of four experiments.

[b] The biochemistry of the malaria parasite. V. Mechanism of pyruvate oxidation in the malaria parasite (Speck *et al.*, 1946).

et al., 1948), but only about 27% by *P. gallinaceum* (Groman, 1951). In actual infections *P. knowlesi* has been observed to destroy 17% of the total hemoglobin when 25% of the erythrocytes were infected; *P. gallinaceum* to destroy only 7.5% of the hemoglobin when 49% of the cells were infected. Cell-free extracts of the parasites hydrolyze hemoglobin (Moulder and Evans, 1946). Such extracts of infected red cells contain a slightly different, and somewhat more active, ribonuclease than do corresponding extracts of uninfected red cells (Miller and Kozloff, 1947).

In view of all these enzymatic activities one wonders why the erythrocytic stages of malaria are obligate intracellular parasites. Perhaps they do not really have so many independent enzymatic activities as we think they do or, perhaps, even though they have such activities they still lack certain essential enzymes or cofactors which they can obtain in nature only from a living host cell. It is an interesting fact that the enzymes of malaria parasites concerned with carbohydrate metabolism are qualitatively identical with those of the host cell. This has been most recently and strikingly noted in a comparison of the respiratory metabolism of uninfected rat reticulocytes with that of reticulocytes infected with *Plasmodium berghei* (Jones *et al.*, 1953; Fulton and Spooner, 1956). Oxidation of pyruvate and the Krebs citric acid cycle were demonstrated in the reticulocytes as well as in the parasites. A comparison of the chemical composition of normal monkey erythrocytes with that of erythrocytes infected with *P. knowlesi* showed the latter to contain 4 times the fatty acids, 3 times the total phosphorus, 3.8 times the phospholipid phosphorus, 2.4 times the 15 minute-hydrolyzable phosphorus, 13.5 times the nucleic acid phosphorus, and 9 times the flavine-adenine dinucleotide McKee *et al.*, 1946; Ball *et al.*, 1948). Except for the nucleic acid phosphorus and the flavine-adenine dinucleotide these increases are small and altogether within a range which could be expected if the increased amounts were a result of stimulated host cell synthesis accompanied by uptake of the products by the parasites. Evidence for such a situation with respect to coenzyme A has already been cited. An entirely similar state may obtain for the enzymes which are present in parasites freshly removed from their host cells. Thus the lactic dehydrogenase of monkey red cells infected with *P. knowlesi* is only twice that of uninfected cells (McKee *et al.*, 1946). There is no proof that the enzymatic activities demonstrated in malaria parasites freshly removed from their host cells are in fact all a result of enzymes produced by the parasites themselves. One is inclined to be especially careful in view of the recent demonstration by electron microscopy that the parasites engulf portions of host cell cytoplasm (Rudzinska and Trager, 1957, 1959), which would consequently still be present in a suspension of extracellular parasites.

A powerful tool for the solution of some of these fundamental problems in the physiology of intracellular parasitism would be the cultivation of a malarial parasite *in vitro* apart from its host erythrocyte. Bass and Johns (1912) first observed the development of human malaria parasites intracellularly in suspensions of intact erythrocytes maintained *in vitro* for a few days. Better results in similar systems have since been obtained with different species of parasites (Ball *et al.*, 1945; Anfinsen *et al.*, 1946; Black, 1946; Glenn and Manwell, 1956; Trager, 1941, 1947a), the best results being those of Anderson (1953) with *P. gallinaceum*. In this work the parasites were kept *in vitro* in a suspension of chicken red cells for nearly 18 days through ten subcultures with a dilution factor of the original culture of 1 : 19,440. Whereas such an *in vitro* system, which is entirely analogous to a virus-tissue culture system, provides a method of great promise for studies in cellular parasitism, it does not help much with the problem with which we are concerned at present. For this problem the parasites must

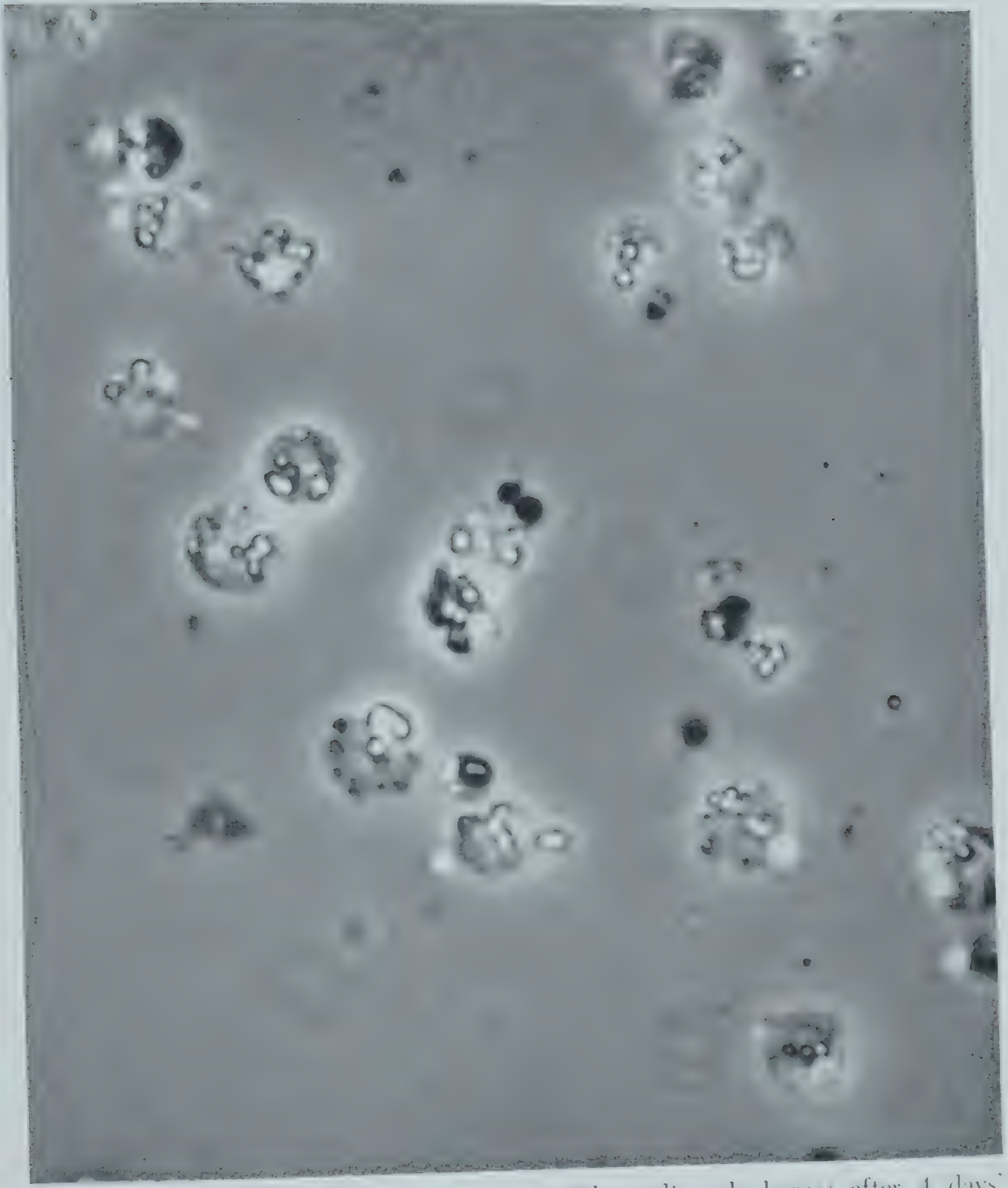


FIG. 8. Extracellular malaria parasites (Plasmodium lophurae) after 4 days' cultivation *in vitro*. Note the cluster of merozoites attached to a refractile residual body. Phase contrast: magnification: ×2480. From Trager (1955); published with the permission of Rutgers University Press.

be maintained in axenic culture—i.e., in the absence of any other living cells. Although this goal has not yet been attained, there has been a partial success with the bird malaria parasite *P. lophurae*. When this organism was removed from its host duck erythrocytes it could be kept alive and developing *in vitro* for periods up to 4 days at 40° C. (Fig. 8).

This extracellular survival and development were favored by duck erythrocyte extract and by the cofactors pyruvate, malate, adenosine triphosphate, diphosphopyridine nucleotide, coenzyme A, and folinic acid (Trager, 1950, 1955, 1957). Malate and chicken erythrocyte extract similarly favored the extracellular development for 1 day of *P. gallinaceum* (Clarke, 1952b). For both species the activity of the red cell extract was nondialyzable (Clarke, 1952a; Trager, 1957). It is interesting to note the requirements for coenzymes of both malaria parasites and rickettsiae. Such a requirement for an external source of coenzymes, which in nature occur chiefly within living cells, could in itself be sufficient to make intracellular parasitism obligatory.

On the basis of the very incomplete information now available we may draw the following picture of the utilization of host cell substances by different types of intracellular parasites. Malaria parasites ingest cytoplasm of the host red cell (Fig. 9) and digest the hemoglobin which it contains, thereby satisfying the bulk of their nitrogen requirement. They utilize carbohydrate and the intermediary metabolites such as pyruvate and malate. They obtain from the host cell a number of coenzymes and certain nondialyzable materials (perhaps enzymes). Rickettsiae secure from the host cell the oxidizable substrate glutamic acid, certain coenzymes, and probably numerous other factors as yet unknown. Bacterial viruses, and perhaps other viruses as well, appear to require much (though not all) of the synthetic machinery of their host cell, redirecting it in such a way as to result in the synthesis of additional virus. It may be remarked that this last statement covers a wealth of ignorance, since synthetic enzymatic mechanisms are as yet relatively poorly understood.

V. Temperature and Intracellular Parasitism

One type of intracellular protozoan parasite illustrates particularly well an apparent relationship of intracellular parasitism to temperature. *Leishmania donovani,* the causative agent of visceral leishmaniasis, develops in its mammalian host as a small, ovoid, intracellular body (leishmania) restricted to the reticuloendothelial cells, particularly of the spleen and liver (see Fig. 6). In its invertebrate vector, a suitable species of sandfly, the parasite multiplies extracellularly as an elongate, flagellated form, the leptomonad. If a suspension of leishmanias from the spleen of an infected animal is left at room temperature many of the organisms transform into leptomonads. These can then be cultured indefinitely in blood agar at temperatures of 25° to not over about 30° C. At higher temperatures the cultures are killed. The reverse transformation, from leptomonads back to leishmanias, does not occur unless the organisms

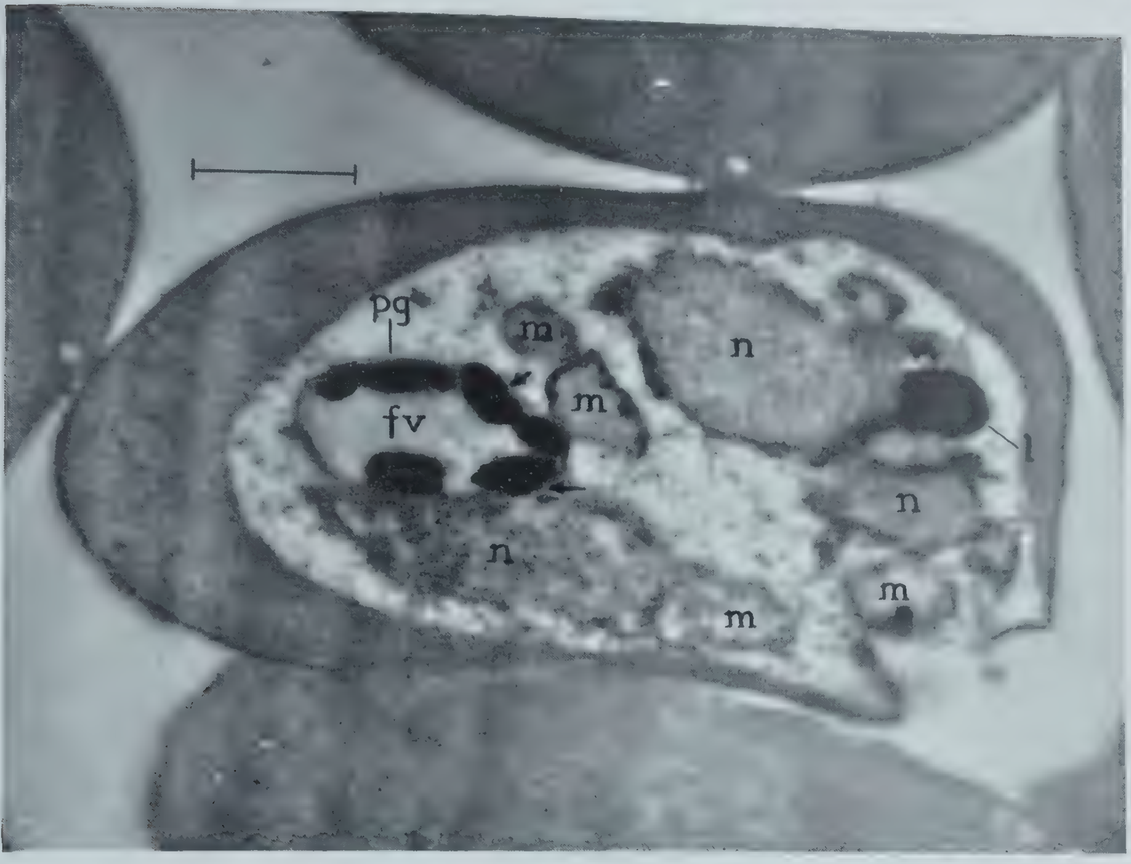


FIG. 9. Plasmodium lophurae within a duck erythrocyte. Electron micrograph of a thin section. The parasite is in a late stage. Note the food vacuole (*fv*) containing large pigment granules (*pg*), the nuclei (*n*), the mitochondria (*m*), and a lipid body (*l*). Magnification: ×16,500. From Rudzinska and Trager (1957); reproduced with the permission of the editor of the *Journal of Protozoology*.

are inoculated into a suitable host or into a tissue culture (Weinman, 1939). In the living cells of the host, or in those of the tissue culture, intracellular development of leishmanias proceeds at 37° C. The extracellular cultivation at 37° of the leishmania form has not been achieved. A limited development *in vitro* at 37° of aflagellate forms intermediate between leptomonads and leishmanias has, however, been obtained in a red cell extract-serum medium (Trager, 1953). It would appear as if the higher temperature imposes on these parasites, perhaps as a result of inactivation of certain synthetic enzymes, a need for external sources of additional growth factors which can be obtained from a living host cell. Similar situations are now known for organisms with simpler nutritional requirements (Borek and Waelsch, 1951; Kasai, 1953), the most interesting being that of the free-living chrysomonad flagellate *Ochromonas malhamensis* (Hutner *et al.*, 1957). Very small increments in temperature above 30° increased the requirements for certain growth factors (as thiamin), introduced new requirements (as for folic acid), and made necessary a much more precise regulation of the proportions of other essential nutrients such as amino acids and trace metals. The story with *L. donovani* will probably turn out to be a more complex one, since even the leptomonads require unknown factors present in red cells, serum, and liver extract (Fulton and Joyner, 1949). Suspensions of leishmanias have a low endogenous oxygen consumption of 7–8 mm.^{3} per hour per 10^8 organisms which is not influenced by glucose or other substrates (Fulton and Joyner, 1949), reminding one of the respiration of leprosy bacilli. Leptomonads from culture, on the other hand, have a low endogenous O_2 consumption of 6–7 which is greatly stimulated by glucose so that it reaches 20–40 mm.^{3} per hour per 10^8 organisms.

A related hemoflagellate, *Trypanosoma cruzi* causing Chagas' disease in man, has a developmental cycle resembling that of *L. donovani.* Here again flagellated extracellular forms occur in the gut of the insect vector (a triatomid bug) and may be readily maintained in culture at temperatures up to about 30° C. Indeed, these flagellates have been grown in a partially defined culture medium (Citri and Grossowicz, 1955). But in the mammalian host, multiplication of the parasite occurs intracellularly, notably in the heart muscle, and the organisms here have the leishmanial form. *T. cruzi* also has in the vertebrate host a trypanosome form, a motile, flagellated stage which does not multiply and is adapted for dissemination from cell to cell and for infection of the insect vector. Interesting observations have been made of the development of this organism in tissue culture (Meyer and de Oliveira, 1948). A 4- to 5-day cycle was noted in infected chick embryo cells in hanging-drop cultures. The cells were invaded by the trypanosomes; multiplication then occurred in the leishmanial form until the cell was filled with leishmanias. In the early stages of infection the host cell continued its activities, including even mitotic division. But when the cell was filled with parasites, degeneration became evident. At this time the leishmanias transformed into trypanosomes and the trypanosomes left the destroyed host cell to seek new ones.

The adaptation to an intracellular mode of life enabled *L. donovani* and *T. cruzi* to overcome the otherwise lethal environmental hazard of the high body temperature of mammalian hosts. Intracellular parasitism in some other forms may well have arisen on this same basis or on the basis of a parellel situation, as for example the protection afforded by the intracellular habitat against toxic constituents of the host's serum (as with leprosy bacilli).

A number of pathogenic fungi exhibit a dimorphism resembling that of *L. donovani.* In the vertebrate host tissues they exist as intracellular yeastlike parasites which multiply by budding as in *Blastomyces* and

Histoplasma or by endogenous spherule formation as in *Coccidioides.* On appropriate solid media at room temperatures they produce a mycelial growth which sporulates. For *Blastomyces* the conversion from yeast type to mycelial type depends solely on temperature and both can readily be grown *in vitro*, the yeast form developing at 37° C. and the mycelial form at 25°. For *Histoplasma* and *Coccidioides* other factors in addition to temperature are concerned (Nickerson, 1951). *Histoplasma* grows in yeast phase at 37° only if supplied with sulfhydryl compounds. Spherule formation *in vitro* by *Coccidioides immitis* has been obtained in a medium of rooster serum plus chick embryo extract (Lubarsky and Plunkett, 1955). It has been suggested that the inability of the dermatophytic fungi to invade deeper tissues may be related to their inability to change to the yeast phase in the presence of bodily conditions which suppress the mycelial phase (Scherr and Weaver, 1953).

VI. REPRODUCTION

The nutrients which intracellular parasites obtain from or through their host cells are used for the growth and reproduction of the parasites. Intracellular microorganisms which belong in taxonomic groupings having extracellular parasitic or free-living forms reproduce in the same way as their extracellular relatives. Thus the symbiotic intracellular yeasts of certain insects reproduce by budding, leprosy bacilli and leishmanias by binary fission. Rickettsiae (Fig. 10) and the viruses of the psittacosis-lymphogranuloma group likewise reproduce by binary fission (Schaechter *et al.*, 1957; Bedson and Gostling, 1954; Weiss, 1955). Malaria parasites and many other Sporozoa reproduce by a process known as schizogony. This is best illustrated by the erythrocytic cycle in malaria. A very small form, the merozoite, which is about 1 μ in diameter and has a relatively large dense nucleus and little cytoplasm, is the only infective stage. In a manner not yet known it penetrates an erythrocyte. Here it increases in size and its nucleus becomes more diffuse. Parasites in this trophozoite stage do not, in nature, leave the red cell. If they were removed artificially they would be unable to initiate an infection. After a period of growth, the nucleus of the parasite begins to divide. When it has produced eight to twelve or more daughter nuclei, depending on the species, membranes are laid down within the cytoplasm so that a merozoite is formed around each nucleus. These daughter merozoites escape to reinitiate the cycle in other cells. One cycle takes 24–72 hours, again depending on the species. The most interesting feature of this cycle is that it includes what the virologists have called an "eclipse period"—a period during which infective stages are not present within the cell. The merozoite which pene-

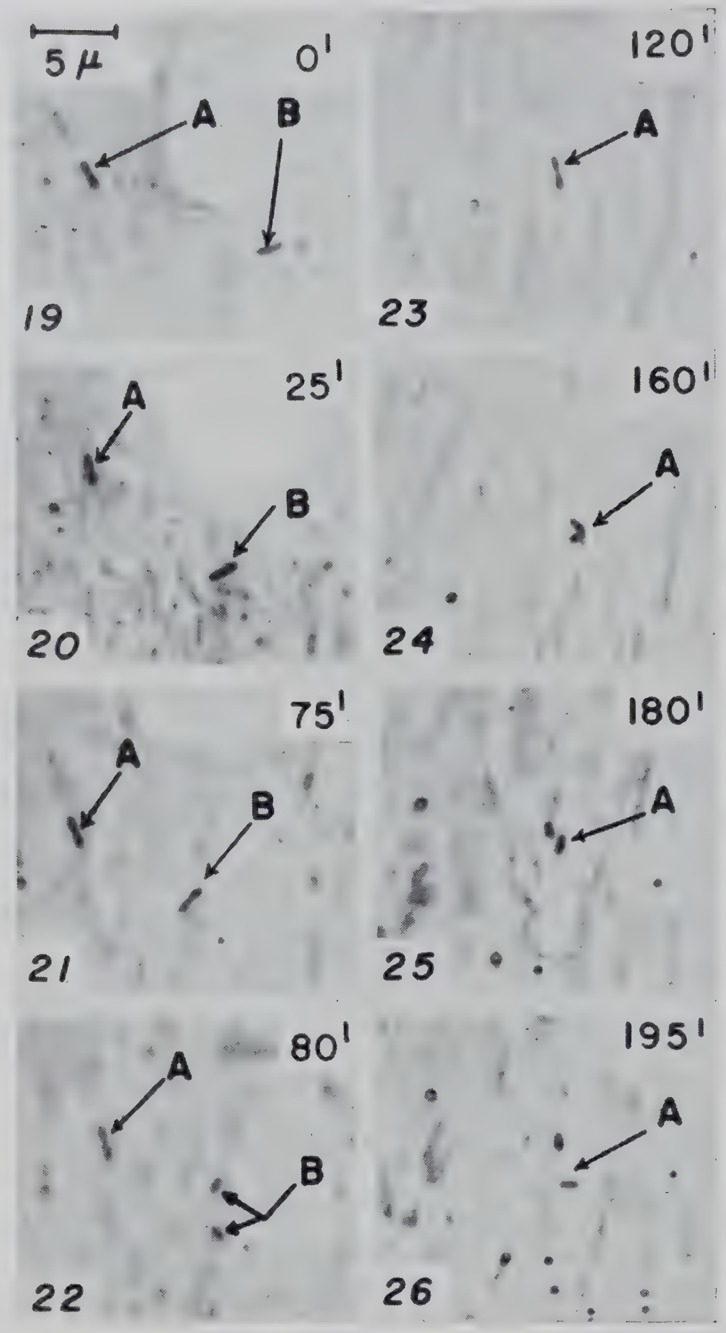


FIG. 10. *Rickettsia rickettsi* undergoing binary fission. Photomicrographs 19–26, taken at the time intervals indicated, of part of the cytoplasm of an infected fibroblast (strain 14 pf) in tissue culture. It will be seen that each of the two rickettsiae *A* and *B* divides into two daughter organisms. During the period between photographs 22 and 23 the daughters of *B* moved away from *A* into another field. Phase contrast photomicrographs; magnification: ×2000. From Schaechter *et al.* (1957), through the courtesy of the authors and with the permission of the editors of *Virology*.

trates an erythrocyte does not give rise to additional infective merozoites until after an "eclipse period" of 24 hours or more. It is evident that the existence of a period when infective stages are not present within the infected cell does not imply anything more than that some kind of developmental cycle different from binary fission is occurring. Independent evidence of some other kind must be provided to show that the infecting particle has lost its identity. Such evidence is available only for the bacterial viruses. Among the large viruses, the "initial body" of the psittacosis-lymphogranuloma group may represent a noninfective developmental stage. Perhaps these viruses undergo processes akin to schizogony, in addition to their reproduction by binary fission (Weiss, 1955; Litwin, 1959). The reproduction of viruses is discussed in detail in Chapter 1.

VII. Escape from the Host Cell

Some intracellular microorganisms never leave the sheltered environment of a living host cell. Thus certain intracellular symbionts of insects are brought by their host cells into the oöcyte of the female host. Here they are subsequently incorporated into special cells of the developing embryo (Buchner, 1953).

Most intracellular microorganisms, however, must leave one cell to invade another. This may be accomplished by destruction of the host cell; bacteriophages and erythrocytic malaria parasites escape at the time of lysis of the host cell. So do numerous other viruses and Sporozoa. Other intracellular parasites are released a few at a time through the surface of the still living intact host cell. This method of release is well illustrated by Eastern equine encephalomyelitis virus (Bang and Gey, 1952) and by rickettsiae (Schaechter *et al.*, 1957). The virus particles or rickettsiae are carried out in fibrils extruded from the cell surface.

Intracellular parasites of multicellular organisms, besides having to get from cell to cell of the same host, must also have means of transmission from one host to another. In this respect they are not different from extracellular parasites; they are transmitted by contact infection, in which case they must have a stage resistant to external conditions; by an intermediate host; or transovarially. These matters are of considerable fundamental interest and practical importance but are outside the scope of the present chapter.

VIII. Effects on the Host Cell

With intracellular parasites which escape from their host cell by its lysis, the end result of the infection is of course destruction of the host cell. This destruction is brought about, however, largely by physiological modifications differing often only in degree from modifications which alter the cell but do not end in its death. A variety of morphological and physiological changes have been observed to accompany intracellular parasitism. Most of these are detrimental to the intact host if not to the infected cell. Certain intracellular microorganisms, however, play an essential role in the economy of their host. These are called symbionts and their association with their host is termed mutualism.

A. Destruction

Bacterial viruses, many viruses of plants and animals, many kinds of intracellular protozoa, rust fungi, produce changes in the host cell which lead to its death and dissolution. The lysis of bacteria by bacteriophages, the cytopathogenic effect of poliomyelitis viruses in tissue culture, the destruction of erythrocytes by malarial parasites or of intestinal epithelial cells by coccidia are particularly good examples. Exactly how the parasites bring about this result is not known. It is not enough to say that the parasite has consumed the interior of the host cell, for this is not always the case. Cells may sometimes be filled with parasites and yet continue for a time to function in a seemingly normal way. With the bacterial viruses and the erythrocytic stages of malaria, since cell destruction does not occur until the infective forms have been produced, it might be that the same enzymatic activities of these forms which enable their penetration into a new cell are also responsible for dissolution of the old host cell. Thus the old host cell would be specifically destroyed by the infective forms of the parasite in order to enable them to reach new susceptible cells.

B. Hypertrophy, Hyperplasia, Neoplasia

Other common readily apparent effects of intracellular parasites on their host cells are stimulation to increase in size, to an increased rate of multiplication, or to both, sometimes followed by neoplasia. Increase in host cell size, accompanied by corresponding hypertrophy of the nucleus, is strikingly illustrated in the infection of ducks by the protozoan parasite *Leucocytozoon simondi* (Huff, 1942). Macrophages, liver cells, and heart muscle cells are invaded by this organism. Even when the parasite is still small the nucleus of the host cell already is hypertrophied. Later, when the parasite is forming a megaloschizont, the host cell and its nucleus become tremendously enlarged, but the characteristic nature of the host cell nucleus is still apparent (Fig. 11). Still later, when the parasite has formed a mature megaloschizont some 150 μ in diameter and containing several hundred cytomeres, the host cell is unrecognizable

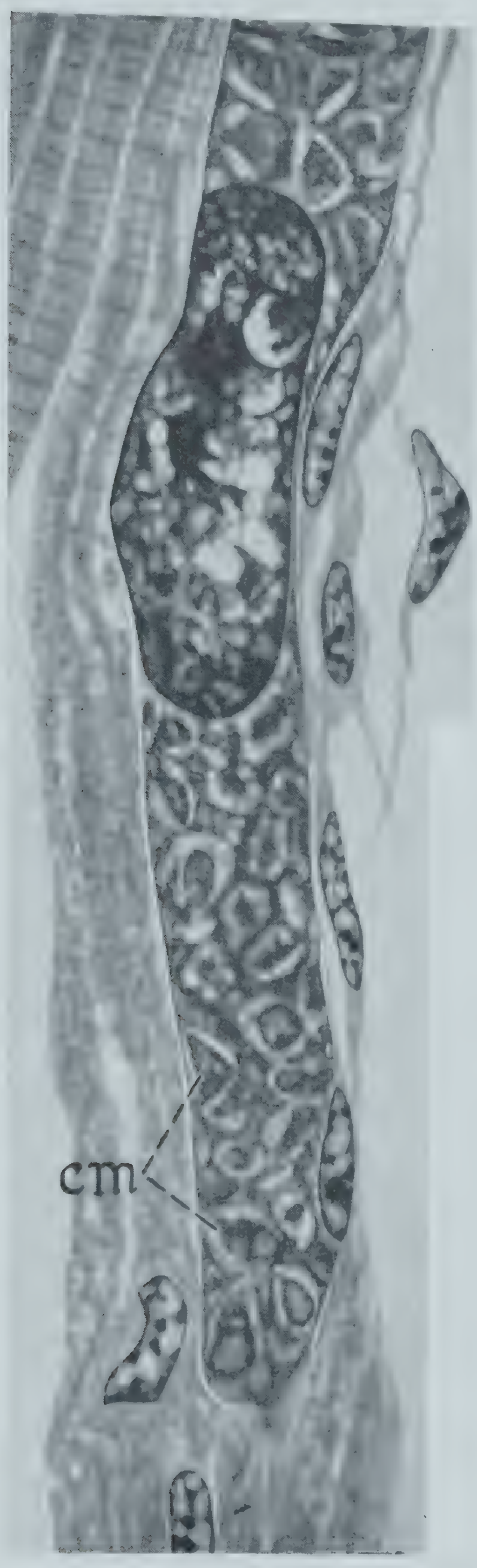


Fig. 11. Greatly hypertrophied cytoplasm and nucleus of a muscle cell infected with *Leucocytozoon simondi* (*cm*, cytomeres of parasite). Note resemblance of structure of hypertrophied nucleus to that of normal muscle nuclei nearby. From a drawing of a section of heart muscle of an infected duck. Magnification: ×1813. From Huff (1942); reproduced with the kind permission of the author and the University of Chicago Press.

and its nucleus is represented by a large structure called the central body. Some have considered the central body to be a part of the parasite (Cowan, 1955), a view which is, however, unlikely in the light of its appearance at earlier stages in the infection of the cell. This tremendous enlargement of the nucleus indicates that the host cell remains alive during most of its hypertrophy. Interestingly enough, the gametocytes of *Leucocytozoon* also cause great hypertrophy of the red blood cells which they invade.

Similar extensive enlargement of host cells occurs in fish in response to infection either with virus of lymphocystis disease (Weissenberg, 1956) or with certain microsporidian protozoa (Weissenberg, 1921; Kudo, 1954). *Glugea anomala*, a microsporidian parasite of the stickleback, produces so-called cysts which consist of enormous cells several hundred microns in diameter, each with a correspondingly enlarged and highly abnormal nucleus. It would be interesting to know the chromosome number and the DNA content of such nuclei, especially at relatively early stages before gross degenerative changes have begun. A significant observation concerning host cell enlargement has been made recently with measles virus propagated in tissue cultures of human malignant epithelial cell lines. The morphological characteristics of the cytopathic effect varied depending on the composition of the nutrient medium used for the cultures (Reissig *et al.*, 1956). When a synthetic medium (Eagle *et al.*, 1957) supplemented with 10% calf serum was used, the infected cells became elongated and spindle-shaped so that the culture appeared as a network rather than a sheet of cells. Omission of glutamine from the synthetic medium induced the formation of many giant multinucleated cells in the infected cultures.

Hypertrophy and also hyperplasia occur in clubroot of cabbage, one of the first intracellular parasitic infections to be studied (Woronin, 1878). The small amebas of this slime mold (*Plasmodiophora brassicae*) are able to penetrate both cell walls and cell membranes of roots and stems of cabbage and related plants. They attack and develop within the nondifferentiated meristematic cells, without at first producing any apparent injury to the host cell (Fig. 12) (Kunkel, 1918). Instead, the host cell is stimulated to excessive growth in size and to excessive cellular multiplication. As a result, the meristematic tissues produce chains of giant cells of no use to the host, instead of the vessels of xylem and phloem required by the growing plant. Hyperplasia of cells other than the giant cells also occurs and large spindle-shaped galls are formed. The ameboid plasmodium eventually kills its host cells and produces masses of resistant spores which are liberated into the soil when the host tissue rots away. It is significant that the stimulus to hypertrophy and hyperplasia travels

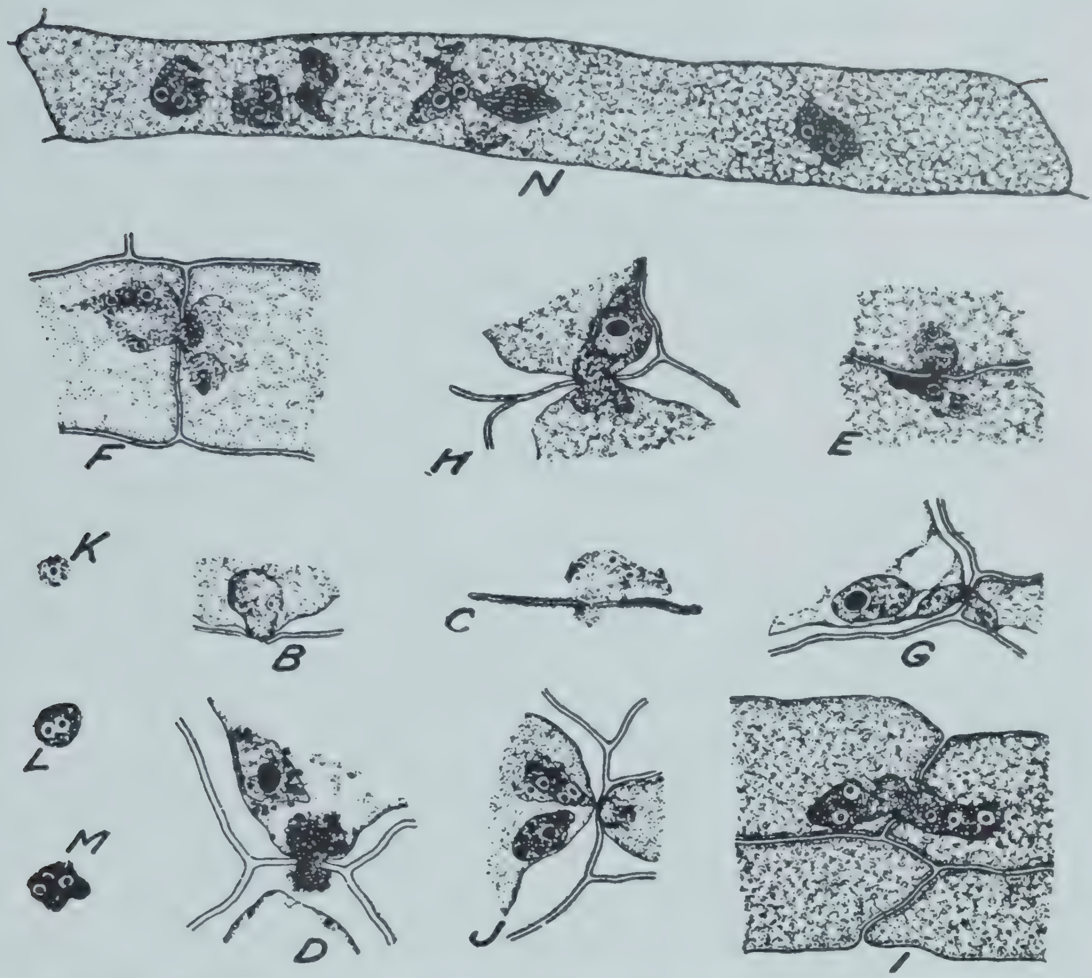


FIG. 12. Plasmodiophora brassicae, the agent of clubroot of cabbage. B, C, D, E, F: Stages in passage of plasmodium through the cell wall. G: A nucleus of a plasmodium passing through the opening in the wall. H: An unusually large opening in the cell wall. I: Plasmodium passing through the end of the cell in the region of the cambium. J: Plasmolysis of the host cells seems to have broken a migrating plasmodium into two parts. K: Ameba from a cambium cell. L, M: Small plasmodia formed in cambium cells far from point of original penetration. N: Infected cambium cell with its nucleus in process of division. From Kunkel (1918); reproduced with the kind permission of the author and the United States Department of Agriculture.

slightly in advance of the invading microorganisms (Kunkel, 1918), indicating the production of diffusible growth-stimulating substances either by the parasite or by the infected host cell.

Tumor formation in plants is brought about also by other agents such as the symbiotic root nodule bacteria (where, however, the growth is of limited size and longevity and serves a useful function for the host plant) and certain viruses, notably the wound-tumor virus (Black, 1945). The hyperplasia, and in a few instances neoplasia, produced in animals by viral infection are discussed fully in Chapter 1, and need not be dealt with further here. The examples already mentioned of hypertrophy and hyperplasia are sufficient to emphasize the fact that such effects may be produced by intracellular infective agents other than viruses. The mechanism of the effects is equally obscure for the larger parasites as for the viruses.

C. Redirection of Metabolism

Hypertrophy, hyperplasia, and even neoplasia may be but particular, morphologically apparent instances of a more general phenomenon—the alteration by the intracellular parasite of the metabolism of its host cell. Only relatively few parasite-host cell systems have been studied in this respect. Interpretation of the results obtained is difficult, since one must decide how much of some additional metabolic activity of the parasite-host complex is contributed by the parasite and how much by an alteration in the host cell. For example, Adler and Ashbel (1940) found that liver and spleen slices of Syrian hamsters infected with *Leishmania* had a high aerobic glycolysis, whereas slices of these organs from uninfected animals had no aerobic glycolysis. Suspensions of washed leishmanias from infected tissues showed anaerobic glycolysis but no aerobic glycolysis. Hence it seems probable that the aerobic glycolysis was an activity of the infected host cells. The possibility remains, however, that it was an activity of the parasites which they could not carry on when removed from the tissues.

We have already indicated how the contribution of a single cofactor by a parasite could be expected to alter greatly the metabolism of a host erythrocyte. The apparent influence of the malaria parasite *Plasmodium lophurae* on the accumulation or formation of coenzyme A has been described in a foregoing section. The CoA content of infected duck red cells was about twice that of uninfected cells and all of the increase could be accounted for in the bodies of the parasites. It has been found recently (Trager, 1959) that the erythrocytes of 2-month-old ducks infected with *P. lophurae* contain ten to twenty times as much folic and folinic acids as those of uninfected birds. In contrast to the findings with CoA, only one-fifth to one-third of the increased folic and folinic acids were present in the bodies of the parasites. The remainder must have been in the infected host cells. Since folic and folinic acids are of importance to malaria parasites, these observations are consistent with the hypothesis that invasion of an erythrocyte by a parasite alters the folic acid metabolism of the host cell in a manner of benefit to the parasite.

Infection with *Rickettsia tsutsugamushi* did not alter the respiratory metabolism of mouse lymphoblast cells in tissue culture (Bozeman *et al.*, 1956).

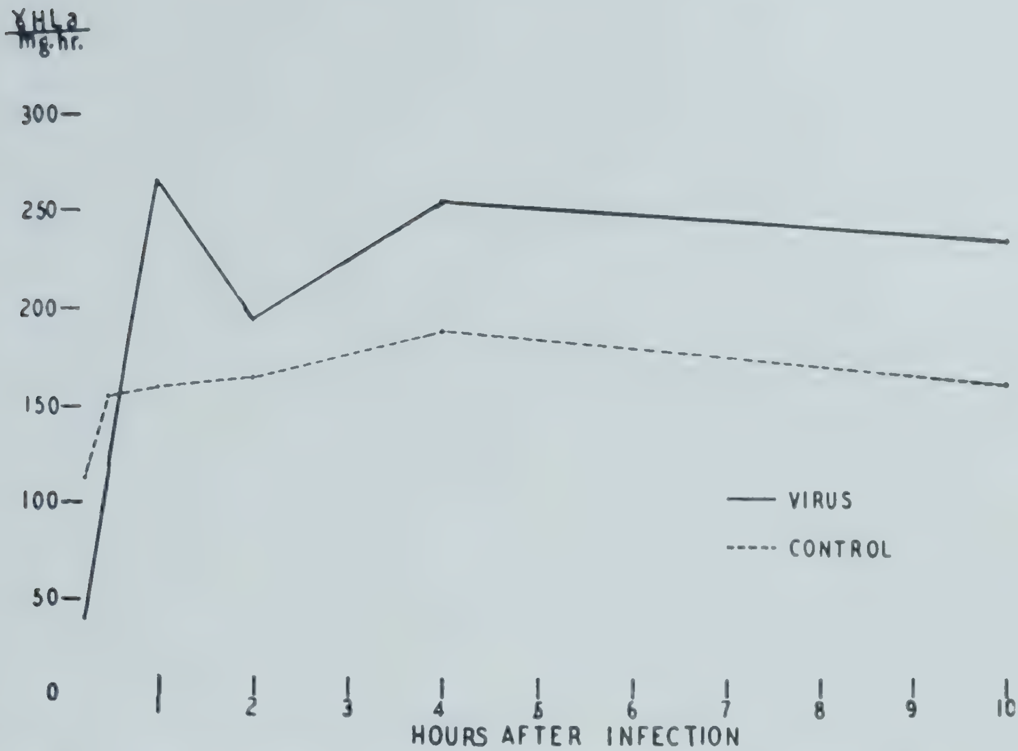


FIG. 13. The effect of poliomyelitis virus on the rate of aerobic lactic acid production of monkey kidney tissue culture. From Levy and Baron (1957); reproduced with the kind permission of the author and the University of Chicago Press.

Since viruses are assumed to have no independent metabolism even when they are actively multiplying within a host cell, differences between the metabolism of uninfected cells and virus-infected cells have usually been ascribed to alterations in the host cell. T1 and T2 phages in *Escherichia coli* B did not affect the oxidation of glucose, lactate, formate, fumarate, and malate, but they did inhibit by 50–80 % the oxidation of pyruvate, succinate, acetate, and glycine (Joklik, 1952). In this connection it is of interest that lipoic acid and pyruvate prevented the induction of lysogeny by ultraviolet in *E. coli* K12 (Borek *et al.*, 1956).

Several neurotropic viruses have been found to stimulate the rate of incorporation of P^{32} into brain tissue (Anderson *et al.*, 1950; Moldave, 1954; Kimura *et al.*, 1955). This may in part at least have resulted from the formation of virus nucleic acid, as has indeed been demonstrated for the uptake of P^{32} by feline pneumonitis virus in chick embryo yolk sac (Zahler and Moulder, 1953). The brains of mice infected with Japanese B encephalitis showed during the course of the virus infection a gradual increase in glutamic acid content to 45 % above normal (Fukushima, 1954).

In monkey kidney tissue culture poliomyelitis virus stimulated both aerobic and anaerobic glycolysis very early in the course of the infection

(Fig. 13) before any new virus had been formed and before any cytopathogenic effects were visible (Levy and Baron, 1957). At the same time the uptake of radioactive glycine was inhibited. The effects were quite different from those of several nonspecific injurious agents. Somewhat similarly, glycolysis by chick embryo chorioallantoic membrane was stimulated by infection with rabbit myxoma virus (Kun and Smith, 1950).

Various enzyme activities have been observed to increase in virus-infected tissues (Bauer, 1947, 1951). Spleens of mice infected with ectromelia had twice the succinic dehydrogenase activity of normal spleens (Latham, 1953). The adenosinetriphosphatase of the muscle tissue of rats infected with Coxsackie virus was markedly increased during the early stages of the disease (Ritis *et al.*, 1953). Chorioallantoic membranes of chick embryos showed increased aldolase activity within 24 hours after infection by any of the following viruses: myxoma, fibroma, Rous sarcoma, swine influenza, Newcastle disease, vaccinia, herpes simplex. This was followed by an elevation in hexosediphosphate dismutation. These changes were not brought about by heat-inactivated virus or by sterile inflammation produced by Celite (Smith and Kun, 1954). The Nadi, dehydrogenase, phosphorylase, and phosphatase reactions were positive in the leaves of virus-infected plants but negative in the leaves of healthy plants (Hirai and Kudo, 1955). On the other hand, the acid and alkaline phosphatase of monkey kidney cells in tissue culture fell sharply during infection with poliomyelitis virus (Kovacs, 1956).

It will be noted that the nature of these changes is not unlike that of the changes found in the few parasite-host cell systems other than viruses which have been studied. With the larger parasites there has been a tendency to ascribe such changes in the metabolism of the parasite-host cell complex to activities of the parasite itself (see, for example, Section IV, B, 1) rather than to alterations in the host cell, a tendency which emphasizes again the inherent difficulty of interpreting measurements made on the cell-parasite complex. A different situation, however, exists with respect to those viruses which reproduce by redirecting the entire synthetic machinery of the host cell in such a way that more virus, rather than host cell constituents, is synthesized. In such extreme parasitism, it may be that the virus polynucleotide competes as a coenzyme with the host genetic coenzyme for the host apoenzymes involved in the synthesis of nucleic acid and protein (Gottschalk, 1957). Of special interest in this connection is the recent demonstration that a bacteriophage containing 5-hydroxymethyl cytosine in its DNA specifically induces in its host the appearance of the enzyme, deoxycytidylate hydroxymethylase (Flaks *et al.*, 1959). This enzyme, not normally present in the host bacteria but essential for the synthesis of the virus DNA, becomes detectable 2 minutes after infection and hence well before virus DNA synthesis begins.

D. *Symbiosis (Mutualism)*

Mention has already been made of the existence of many kinds of nonpathogenic intracellular microorganisms. Such organisms are assured a protected ecological niche with a certain and abundant food supply. Since some of these microorganisms occur regularly in every individual of the host species and since the host often is specially adapted for their maintenance and transmission, it has seemed likely that the association is of benefit to the host as well as the parasite; such an association would be an example of mutualism and such a parasite would be termed a symbiont.

There are four main categories of suspected or demonstrated mutualism between intracellular microorganisms and their hosts: (1) algae in aquatic invertebrates; (2) fungi (mycorhiza) in plants; (3) bacteria (rhizobia) in leguminous plants; (4) yeasts and bacteria in invertebrates, particularly insects.

1. *Algae*

Intracellular algae are almost wholly restricted to those invertebrates which digest intracellularly. The associations may have arisen with acquisition by the algae of the ability to resist digestion. Their significance to the host animal varies widely and is only rarely a true mutualism (Yonge, 1944). Early work with forms such as the ciliate *Paramecium bursaria* and the coelenterate *Chlorohydra* showed that these organisms, which contain green zoochlorellae, can survive in oxygen-poor water for much longer periods than closely related species not containing algae (Wilson, 1891; Pringsheim, 1928). It seems doubtful, however, whether this is of much importance under natural conditions (Yonge, 1944). The removal of the waste products of animal metabolism by the algae may improve the metabolic efficiency of the host; this might be an advantage under conditions of severe competition such as obtain in tropical waters. Similarly, the algae in tropical waters where supplies of nutrient salts are limited would derive a considerable advantage from close association with a host animal. The cultivation *in vitro* of some of the zooxanthellae should help to illuminate these interesting problems (McLaughlin and Zahl, 1957).

Direct nutritional benefit derived from the algae by the animal host has been clearly demonstrated in several instances. The giant clam *Tridacna* has become specialized in such a way that the edges of the mantle are exposed to the sunlight and contain masses of zooxanthellae lying within blood cells. The blood cells migrate from the mantle edge to the region of the digestive diverticula and there digest the zooxanthellae which have previously flourished within them (Yonge, 1944). Perhaps the lack of light in the new situation permits this to occur. In any case we have here the maintenance by each animal of an algal farm upon the produce of which it feeds. The association is a more subtle one in the marine turbellarian *Convoluta roscoffensis.* Here the algae are passed in the egg case, so that the young become infected when they hatch. The organs of feeding later degenerate and the animal lives presumably at the expense of materials produced by the contained algae. Eventually the algae themselves are digested, except for those which infect the egg case, and the host dies after having laid its eggs (Yonge, 1944).

The nutritive relations between symbiotic algae and their host have been best shown experimentally for *Paramecium bursaria* (Pringsheim, 1928; Loefer, 1936a, b). Although this ciliate in nature is mixotrophic and feeds on bacteria, it can live axenically in simple media, even under strictly autotrophic conditions, if kept in the light. Other species of *Paramecium* which do not contain zoochlorellae, or *P. bursaria* if deprived of zoochlorellae, will not grow free from living bacteria unless provided with a complex nutrient medium containing an array of essential amino acids, vitamins, purines, pyrimidines, a sterol, and an as yet unidentified nondialyzable factor from yeast (Johnson and Miller, 1956). Presumably the zoochlorellae can supply *P. bursaria* with these nutrients.

2. *Mycorhiza*

A majority of wild perennial plants have fungi associated with their roots (Gwynne-Vaughn and Barnes, 1930). The nutritive relations between the fungi and the host plants vary by small gradations from purely mechanical effects through pathogenic parasitism to mutualism (MacDougal and Dufrenoy, 1946). Some of the main components of protoplasm are elaborated by the fungi of certain orchids and by mycorhiza of pine, and are translocated to the cortex of the host. The minute seeds of terrestrial orchids fail to germinate unless sown together with fungus from roots of the plant. Hyphae of the fungus invade cells of the embryo. Rounded wefts of hyphae also occur in cortical cells of the young roots of orchids and seem to be essential to growth of the host plant. In some species loss of roots and reduction or loss of photosynthetic mechanisms have occurred, so that the plant receives all its nutrients from the mycorhizal rhizomes. There is good evidence that the fungi from plants of the Ericaciae and Orchidaciae are able to fix atmospheric nitrogen and to provide their host with utilizable nitrogen compounds (Foster, 1949).

3. Rhizobia

In the United States alone the quantity of nitrogen fixed by the root nodule bacteria of leguminous plants in one year has been estimated at 5–6 million tons, as compared to only 0.5 million tons in industrial fertilizers and 2–3 million tons in manure (Virtanen, 1947). Even more remarkable are the physiological relationships between these intracellular bacteria and their host plants (Virtanen, 1947; Allen and Allen, 1954). Infection of the legume is effected by rhizobia present in the soil and occurs usually via the root hairs. The exact mechanism of entry is not known, but almost immediately after the root hair has been invaded the rhizobia become aligned into a hyphalike thread which proceeds directly towards the base of the hair. In a day or two this infection thread reaches the inner wall of the epidermal cell and enters the root cortex, where it branches. Intracellular invasion of the cortical cells occurs, the rod-shaped bacteria being at first confined in a slimelike matrix. Later the bacteria are released into the host-cell cytoplasm and both the cell and its nucleus enlarge considerably. Adjacent cells also enlarge, and both infected and uninfected cells divide rapidly. The infected cells become packed with rhizobia which now assume various involution forms; these are the bacteroids. The resulting nodule consists of an outer cortical uninfected layer beneath which lies the nodule meristem. This is composed of small, compact, uninfected cells which give rise to new cortex on one side and to new parenchymatous infected cells of the bacteroid zone on the other side. The infected cells have a chromosome number twice that of the uninfected cells and are four to eight times larger. The bacteroidal zone is the seat of symbiotic nitrogen fixation. It is also unique in that it contains the red pigment leghemoglobin (see Thorogood, 1957) and the associated pigments legcholeglobin, legmethemoglobin, and coproporphyrin. The nodules of annual legumes have a relatively short life; those of woody plants may persist for a time, but they too eventually undergo necrosis and slough off. Their fate is thus very different from that of the pathological growths produced by the closely related crown gall bacteria (*Agrobacterium tumefaciens*).

The fixation of atmospheric nitrogen into compounds utilizable by the host plant occurs in the nodules whenever a suitable host is infected by an appropriate strain of *Rhizobium leguminosarum*. There are strains of bacteria which form nodules but do not fix nitrogen. A strain effective in one plant may be ineffective in another (Virtanen, 1947). Periods of activity of the nodules correspond to periods of active growth of the plant. There are striking differences in size and weight between uninfected and infected plants when both are grown on a nitrogen-deficient medium, the latter being much larger. Nitrogen fixation by the nodules is almost always correlated with the presence of the bacteroidal (rather than the rod-shaped) forms, and always with the presence of leghemoglobin. It is very significant that neither the plants nor the rhizobia produce hemoglobin independently—only during the stage of effective symbiosis is the hemoglobin formed. Here is another instance of a physiological modification brought about by intracellular parasitism. Only when infected by a particular kind of parasite do the host cells, or perhaps the complex of host cell plus parasite, synthesize hemoglobin. The hemoglobin evidently plays an essential role in nitrogen fixation, but what this is remains unknown.

Although the rhizobia can be readily cultivated *in vitro,* such cultures have in general not been observed to fix nitrogen. Recently, however, Heumann (1952) has obtained nitrogen fixation *in vitro* by certain strains grown on a medium with 15% human blood. It is worth pointing out that this one association between rhizobia and legumes includes nearly all the physiological problems of intracellular parasitism which have been discussed in this chapter: host-parasite specificity, penetration of the host by the parasite, metabolic interactions between host and parasite including hypertrophy and hyperplasia (though limited) of the host cells and the production of specific substances not normally formed by the host cells (hemoglobin).

4. *Symbionts of Insects*

Bacteria and yeasts of special kinds occur regularly in association with many different species of insects. Most of these are intracellular and nonpathogenic. They are restricted to certain regions of the host's body, being contained within specialized cells (mycetocytes) or even within the cells of special organs (mycetomes). The microorganisms occur especially in insects which live on restricted diets such as wood, blood, or plant sap. Particularly striking is the fact that many diverse specializations have been developed by the host, all designed to insure transmission of the symbionts from generation to generation of the host (Buchner, 1953). The view that the association between these microorganisms and their insect hosts is a mutualistic one has received strong support from experimental work of recent years (Koch, 1956; Richards and Brooks, 1958).

Some of the beetles which live in flour and other stored products harbor yeastlike symbionts within enlarged cells of diverticula of the midgut. In adult females the symbionts also occur extracellularly in a pair of tubules which open into the vagina. As each egg is being laid, its surface is smeared with the contents of the tubules. The yeasts, which are contained in a special secretion, can evidently withstand drying. When the larva hatches it eats a portion of the egg shell and thereby becomes infected with the yeast.

Koch (1933) was the first to take advantage of this relatively simple method of transmission to secure symbiont-free larvae of the beetle *Stegobium paniceum*. Immersion of the eggs for 2 minutes in a solution of 5% chloramine in 70% alcohol killed the yeasts on their surface. Larvae hatching from such eggs were aposymbiotic. They grew very slowly on a diet of pea sausage which was altogether adequate for the rapid growth of control normally infected larvae. If the symbiont-free larvae were fed a diet of pea sausage plus 10% dried yeast, their growth was nearly as good as that of normal larvae. Evidently the symbiotic yeasts supplied their hosts with growth factors and in this way permitted them to grow on an otherwise deficient diet. The particular growth factors supplied by the symbionts have been determined for *Stegobium paniceum* and for the related form *Lasioderma serricorne* (Blewett and Fraenkel, 1944). Both these species of beetles when infected with their natural symbionts grew normally on vitamin-deficient synthetic diets which did not support growth of the symbiont-free larvae or of other species of flour beetles, such as *Tribolium confusum*, which never have symbionts. It was found that the symbiotic yeasts furnished their hosts with biotin, pteroylglutamic acid, thiamin, riboflavine, nicotinic acid, pyridoxine, pantothenate, choline, and an essential sterol. The yeasts of both *Lasioderma* and *Stegobium* were grown in culture and shown to behave as different species. Symbiont-free larvae of each species of beetle would grow normally on the deficient diet if reinfected with either its own species of yeast or that of the other species (Pant and Fraenkel, 1954). The mutualistic nutritional relationship of these insects and their intracellular microorganisms has here been not only demonstrated beyond the shadow of doubt, but also worked out in detail.

The intracellular microorganisms of roaches present a more complicated problem. Morphologically they appear to be bacteria. They occur in specialized mycetocytes scattered in the fat body (Fig. 14) and also in cells adjoining the ovarian tubules. From the latter they come to surround the oöcyte and eventually to penetrate within it. During embryonic development the bacteria are carried to the region of yolk cells in the center of the embryo, and by the time the young roach is ready to hatch they are already localized in the mycetocytes lying within the lobes of the fat body. It is at once apparent that breaking the chain of infection is here a much more difficult matter than with the beetles discussed above. The first success was obtained by Glaser (1946). Prolonged treatment of adult roaches (*Periplaneta americana*) with sulfa-

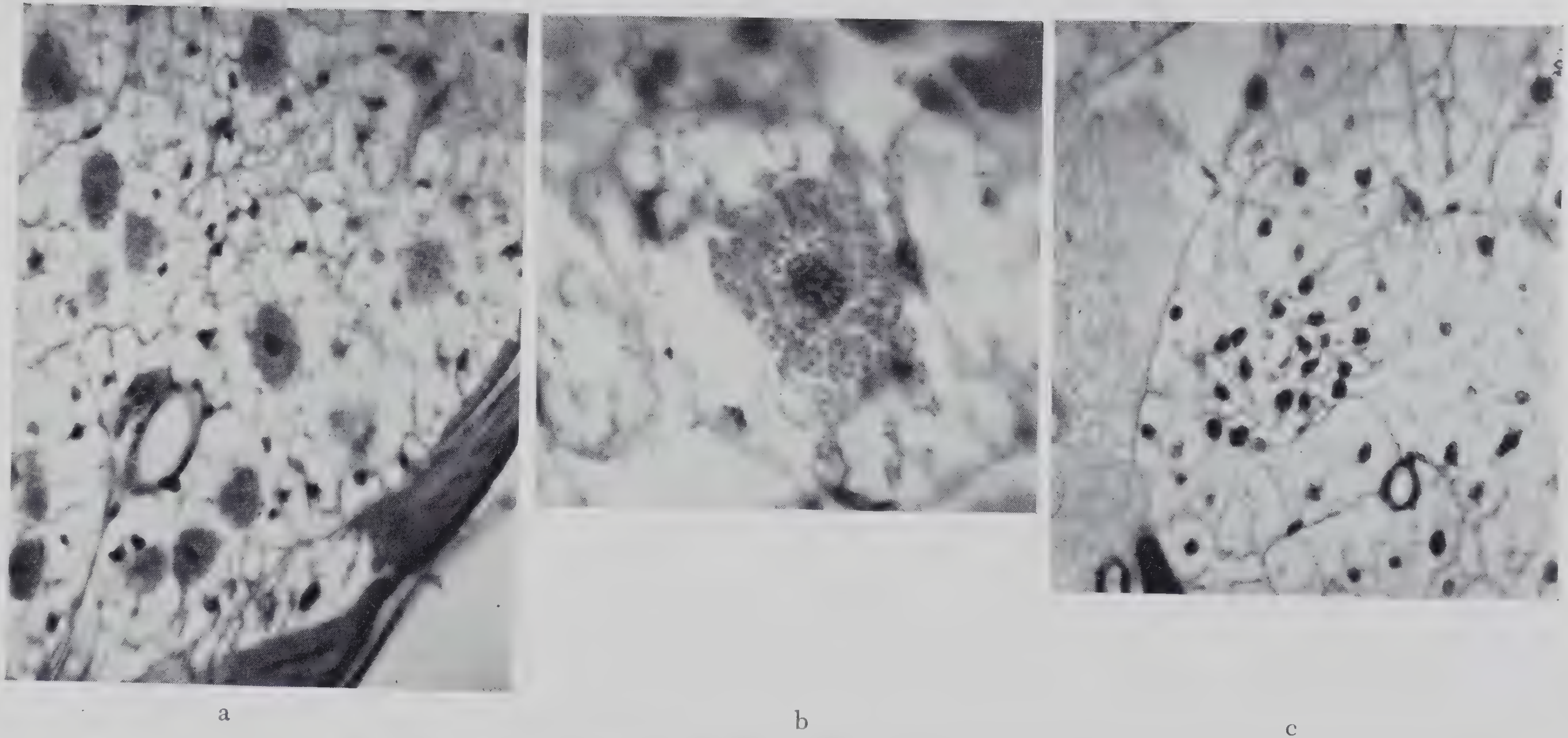


FIG. 14. Symbionts of the roach *Blatella germanica* as seen in stained sections. a. Fat body of normal roach showing mycetocytes with symbionts; magnification: ×211. b. A mycetocyte showing the cytoplasm filled with bacteria; magnification: ×704. c. Fat body of aposymbiotic roach showing small, empty mycetocytes; magnification: ×211. (From photographs kindly supplied by Dr. Marion Brooks, University of Minnesota.)

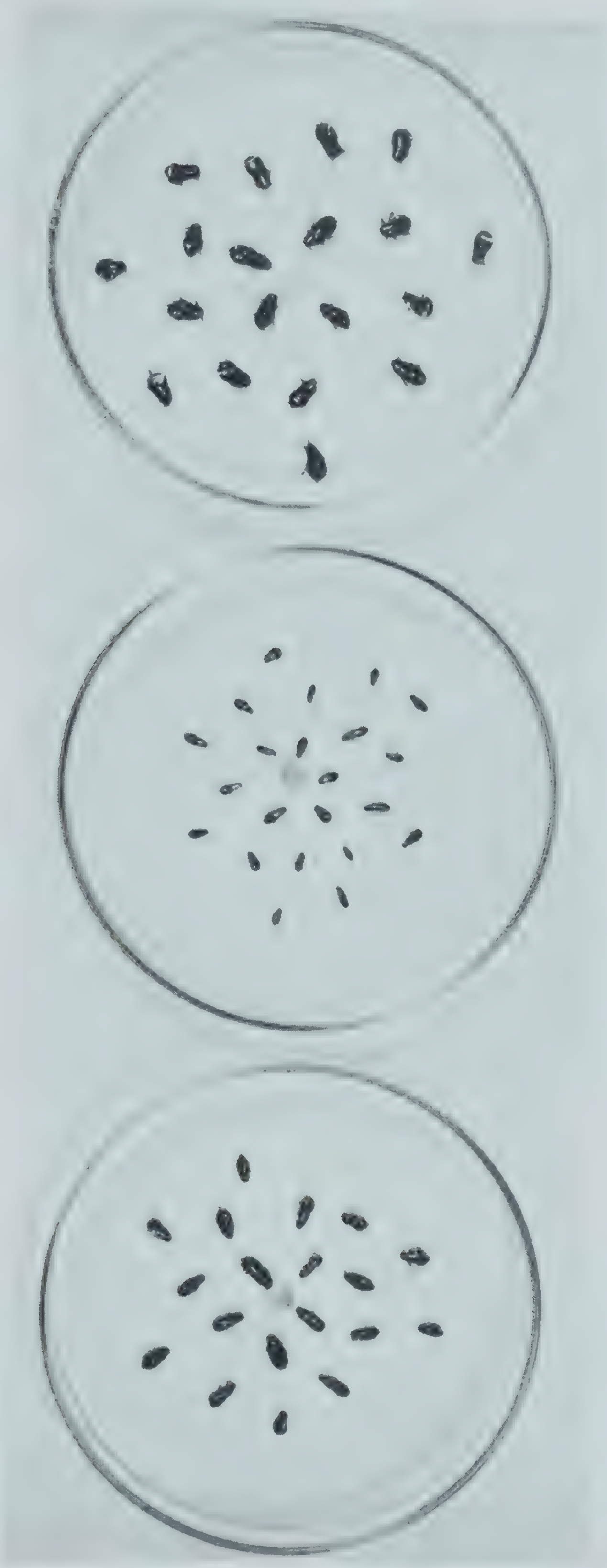


FIG. 15. *Roaches* (*Bratella germanica*) 40 days old. Top group: normal, fed ground dog biscuit. Middle group: aposymbiotic, fed ground dog biscuit. Bottom group: aposymbiotic, fed ground dog biscuit plus 25% yeast. These specimens were supplied through the kind cooperation of Dr. Marion A. Brooks, University of Minnesota. Photo by J. A. Carlile.

thiazole, briefer treatment with penicillin or with exposure to a high temperature (39° C.), all gave a total of six males and seventeen females which survived the treatment and were free from symbionts. These insects behaved normally and the males showed normal sperm production. But ovaries were absent from the females. Similar results have been produced by treatment of the oriental roach *Blatta orientalis* with antibiotics (de Haller, 1955; Frank, 1956). Roaches rendered symbiont-free early in life took twice as long to mature as normal roaches, were considerably smaller, of a lighter colour, and showed other signs of avitaminosis despite the fact that they were fed an enriched diet. In this work, as in Glaser's, the effects of aposymbiosis were observed on the same individuals which had received the treatments responsible for sterilization, so that the possibility remained that these treatments might have been directly responsible for the deleterious effects. This possibility was eliminated in the striking experiments of Brooks and Richards (1955), who prevented the transovarial inheritance of symbionts in cockroaches (*Blatella germanica*) by feeding the parent roaches a diet of ground dog biscuit plus 0.1% Aureomycin. The symbiont-free eggs so obtained produced symbiont-free nymphal roaches never subjected to Aureomycin. When these roaches were fed ground dog biscuit, a diet perfectly adequate for the growth of normal roaches, they were practically incapable of growth (Fig. 15). If the aposymbiotic roaches were fed dog biscuit plus 25% yeast they grew slowly (Fig. 15), and reached maturity in two to three times the period required by normal roaches. Adult aposymbiotic male and female roaches obtained in this way did not reproduce as well as normal roaches, but an aposymbiotic colony could nevertheless be maintained on the high yeast diet. Symbiont-free roaches of the second and third generations were like those of the first generation.

The symbiont-free *B. germanica* could not be reinfected by the feeding or injection of suspensions of symbionts prepared from normal roaches. If they were implanted in the hemocoele with pieces of normal *B. germanica* fat body containing cells with bacteria, the introduced bacteria remained confined to the implanted tissue (Brooks and Richards, 1956). Roaches so treated, however, showed a gradually accelerating growth rate. With the roach, as with the beetles, the symbionts appear to furnish accessory growth factors which can be more or less replaced by a diet high in yeast. The symbionts of roaches may also be concerned with the disposal of waste products, for in both *Blatta orientalis* and *Blatella germanica* aposymbiotic individuals had larger accumulations of urate crystals than did normal roachs of the same age.

A situation of still greater complexity is presented by the blood-sucking louse (*Pediculus corporis*) and its relatively large symbiotic bacteria. In this species, as in roaches, infection is transmitted by way of the ovaries. During embryonic development the bacteria are confined in a group of cells which come to lie in the lumen of the developing alimentary tract. These cells degenerate with liberation of the bacteria, which then infect the cells of an outgrowth of the alimentary tract. This outgrowth becomes separated from the gut and forms a special structure the mycetome, a conspicuous organ which lies near the ventral surface of the body. In adult males the mycetome and the contained bacteria degenerate. In adult females the bacteria leave the mycetome (which later degenerates) and infect cells of the ovarial ampullae, from which in turn infection of the developing oöcytes occurs. Removal of the mycetome could be effected by operative procedures or by centrifugation of the eggs (Aschner and Ries, 1933; Aschner, 1934). Extirpation of the mycetome from adult females in which the bacteria had not yet migrated to the ovarial ampullae was followed by grave deficiency symptoms and inability to reproduce, whereas the operation had no effect if performed after migration of the bacteria. Younger stages rendered symbiont-free failed to grow even if fed by rectal injection of blood. The deficiencies could be partly overcome by the injection of yeast extract. In an extension of this work Puchta (1955) has succeeded in bringing a few symbiont-free lice through to the adult stage by feeding them hemolyzed blood plus a mixture of the B vitamins. It is a striking fact that insects which feed throughout the life cycle exclusively on blood (well known to have relatively low concentrations of most vitamins) all contain symbiotic bacteria. This is true of the bugs such as *Cimex* and *Rhodnius,* of the bloodsucking lice, of Diptera such as the sheep ked *Melophagus* and the tsetse fly *Glossina.* On the other hand, forms which take blood as adults but as larvae have a diet in which microorganisms are abundant (e.g., mosquitoes, tabanid flies) do not contain symbionts. In lice, as in roaches and certain beetles, the main benefit (and the only one clearly demonstrated in experiments) conferred by the symbionts on the host is the supplying of growth factors which permit the host to live on otherwise inadequate diets. It is not hard to imagine, however, quite different roles for some at least of the myriads of kinds of insect symbionts. The decomposition of waste products, with perhaps their synthesis into compounds useful to the host, and nitrogen fixation, are among the possibilities. There is as yet no good evidence for either of these activities. The symbionts of certain aphids have been said to be capable of nitrogen fixation, but no uptake of N^{15} by the intact aphids could be found (Smith, 1948).

Although the foregoing discussion of symbiosis has been included as an aspect of the effects of intracellular microorganisms on their host cells, it must be emphasized that the beneficial effects of the symbionts are exerted primarily on the host as a whole, and only indirectly, and often temporarily, affect the host cell itself. The host cells of symbiotic microorganisms, like those of some pathogenic intracellular parasites, are abnormal cells, usually considerably hypertrophied, whether they are plant cells in a root nodule or insect cells in a mycetome. The symbiotic microorganisms and their host are so well adapted to each other that neither is capable of destroying the other and each receives benefit from the other. It would be reasonable to expect associations of an intermediate grade in which the host can control the parasite and is neither injured nor benefited by it. An understanding of the mechanism of such control would be of fundamental significance and considerable practical importance. What is latency if not a period during which the host exerts such a control on the parasite? Little is known of these innate controlling mechanisms. That they may be in delicate balance is suggested by the recent findings that certain metal deficiencies in the diet of the roach *Blatella germanica* lead to a situation in which the ordinarily symbiotic bacteria become harmful to their host (Brooks, 1957).

IX. General Remarks

A better understanding of intracellular parasitism may be expected to develop concomitantly with better understanding of cellular physiology in general. Each field of study will continue to draw from and contribute to the other. Certainly a knowledge of the biochemical basis for host-parasite specificity at the cellular level would at the same time give very important information concerning the subtle structure and materials which distinguish different kinds of related cells. Detailed study of how a parasite alters the metabolism of its host cell might illuminate the whole problem of control of cellular metabolism.

Particularly valuable might be a knowledge of the special nutritional factors which intracellular parasites derive from their host cells. Since obligate intracellular parasites have not been cultured apart from their host cells and will not grow in media containing all the known growth factors for free-living organisms plus extracts of natural materials, the assumption seems justified that the parasites require as yet unknown, perhaps relatively labile, constituents of the host cell. Just as free-living organisms of various kinds, from bacteria to rats, served as indicators for the discovery of vitamins and other growth factors, each of which opened a whole chapter in cellular biochemistry, so it seems likely that intracellular parasites will serve as indicators of additional substances of great physiological significance—substances which most cells can make for themselves but which intracellular parasites must obtain preformed from their host cells.

Physiological studies with intracellular parasites should point the way to knowledge of the intracellular milieu within which the organelles of the normal cell perform their varied activities. It is already clear that the morphology and the physiological activities of isolated nuclei and mitochondria are profoundly and rapidly affected by the nature of the medium in which they are suspended (Anderson and Wilbur, 1952; Watanabe and Williams, 1953; Philpot and Stanier, 1955; Birbeck and Reid, 1956; Fonnesu and Davies, 1956). The activities of intracellular enzymes are influenced by the ionic environment (Hastings, 1940; Ashwell and Dische, 1950), a solution with high K^+ being essential for glycogenesis by rat liver slices (Buchanan *et al.*, 1949). Similarly, solutions with high K^+ and low Na^+ produced less injury than solutions with high Na^+ when injected into the plasmodium of the myxomycete *Physarum* (Chambers, 1943) or into the cytoplasm of two species of amebas (Kassell and Kopac, 1953). The salivary gland chromosomes of *Chironomus* larvae retained their natural appearance best in a solution with relatively high K^+ and low Na^+ (Chambers, 1943). It is interesting that the extracellular survival of a malarial parasite has been observed to be favored by a medium of high K^+ content (Trager, 1941) and that solutions with high K^+ have been useful in the isolation of rickettsiae (Bovarnick and Snyder, 1949). Possibly the favorable effect which gelatin has on the extracellular survival of malaria parasites (Trager, 1950) rests on a basis similar to that responsible for the favorable effects of pectin (Brown, 1951) and gum arabic (Dounce and Litt, 1952) on isolated nuclei and of dextran on isolated mitochondria (Birbeck and Reid, 1956). The available facts are as yet fragmentary and inconclusive, but they strongly indicate that further progress in this field will be fundamental to a complete understanding of what goes on in a living cell.

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CHAPTER 5

The Neuron

By H. HYDÉN

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INTRODUCTION

The following review discusses the current knowledge of the morphology, chemistry, physical properties, and some functional aspects of the neuron. Such data should be correlated to give a synthesis of this functional unit; the information seems, however, to be insufficient—at least the task is a Utopian project for the author.

Among cells, the neurons are extraordinarily difficult to study under conditions similar to those *in vivo* without interfering with the subject. Transferred to a cytodimension, the effect of a Heisenberg's uncertainty principle seems to prevail. In ordinary cytological preparation procedures, a large part of the organic material is removed from the nerve cells. One consolation is that the Nissl pattern offers a reproducible picture in cytology. From a chemical point of view, however, only a skeleton is left of the nerve cell in a histological section. An exposed nerve preparation for recording impulses, is, furthermore, hardly in a physiological condition. The cell culture technique promises more and permits direct observations, but has evident drawbacks.

Certain problems related to the cytophysiology of the neuron will be considered. The volume of the neuron can change considerably, e.g., rapidly by uptake of water, and within days owing to the synthesis of proteins and lipids. At high resolution the nerve cell cytoplasm shows structures typical also for protein-producing glandular cells. This fits with well-established evidence for a steady nucleoprotein production which increases at increasing neuronal function and with indications for a high nucleoprotein, functional metabolism. A tentative explanation as to why the neuron produces nucleoproteins when functioning is discussed in Section VI.

Many primary questions can hardly be more than touched on at present. Are there any objective expressions for a biochemical specificity between neurons? Are the electrical impulses, propagated and transmitted by the neuron, features of primary significance or by-products and phenomena of secondary rank? Nevertheless, technical advances have begun to make it possible to tackle such problems.

Another problem is the regeneration of the peripheral neurons which can occur repeatedly, and the importance the periphery may have as a feed-back mechanism for functional reconstitution. The chapter ends with some thoughts on an intraneuronal memory mechanism, based on a principle anchored in the genetic mechanism of the cell and starting from the fact that memory or change by use is a phenomenon of wide scope in nature.

A. *Observations in Nerve Cell Cultures*

Recent developments of the technique for studying living cells in cultures have been applied also to nerve cells and glia cells. The results considerably increase the sparse data of nerve cell in regeneration, but also open up a new field which seems necessary and fruitful for the study of the dynamic activity of the neurons *in vivo* (Harrison, 1910; Lewis and Lewis, 1912; Lewis, 1931, 1945, 1950; Speidel, 1933, 1950; Pomerat, 1951, 1955; Pomerat *et al.*, 1957; Nakai, 1956; Hughes, 1953; Peterson and Murray, 1956; R. S. Geiger, 1957).

Lively movements of the nerve cell processes during formation have been observed (Murray and Stout, 1947), as well as a slow rotation of the nucleus, pinocytosis and vacuolar ascent in the axon, and undulating movements of the cytoplasmic edge and of thin membranes on the neurite (Pomerat *et al.*, 1957).

The nucleus was seen to change its shape, and nucleolar material to pass through the nucleoplasm into the cytoplasm (R. S. Geiger, 1957). Mitoses of cortical neurons were reported to occur frequently enough in the cultures to be a characteristic phenomenon (R. S. Geiger, 1957). If further studies could reveal how to induce neuron mitosis also *in vivo*, this observation would indeed represent a landmark within neurobiology. There occurred a streaming of cytoplasm throughout the perikaryon and the processes, and a slower movement of granules to and from the nuclear membrane.

When formation of synapses occur in the culture, the processes show rapid movements at the point of contact, and the mitochondria within become very motile.

Furthermore, the intricate relationship between nerve cells and glia cells was reflected by the rhythmic contractions of the glia cells and their fragile, undulant membranes. This activity was thought to exert a rhythmic pressure on the neurons and to facilitate intra- and extraneuronal fluid movements (see Pomerat, 1951).

B. *Quantitative Problems*

The structures which compose the nervous tissue are all intricately built. This is clearly seen from sections stained by different methods, all of which bring out some characteristic details. The finding that the composition of the nervous tissue is still more complicated if a lower dimension is approached, is a successful result of use of the electron microscope. The three-dimensional structure of the nervous system is, in fact, enormously complicated. Any method of analysis the aim of which is to reveal the distribution and give a quantitative assay of chemical components in relation to structure must have a high resolving power if it is to be successful. This partly explains why so many fundamental neurobiological data are still not available. It is difficult to reconcile information obtained at different levels of dimensions, into a synthesis of the neuron at an *in vivo* level, to paraphrase Hegel.

Some problems of this kind are discussed by Pope (1955). Quantitatively, how much does each element of a sample of nervous tissue and its processes contribute to the volume, the mass, the total solids, and the cellular surface area? How much valid information can be obtained from a biochemical analysis of a milligram of nervous tissue if the contribution of each cellular species to the measured concentration is considered? Is such a chemical result best expressed in terms of wet weight, unit volume, dry weight or in relation to protein or deoxyribonucleic acid (D.N.A.)?

Sampling. The sampling of nerve tissue components for biochemical or physical analyses on macro- down to ultramicroscale is of decisive role for the conclusions. Differentiated centrifugation of homogenates according to various methods has been used for studies of milligram samples (Richter and Hullens, 1950; Chu, 1954). The fractions are, however, not satisfactorily pure. The best results are obtained with nuclei. For histochemical studies Lowry (1952), Pope (1952, 1955), and Robins *et al.* (1956) use sections comprising structurally well-defined layers of the central nervous system (CNS) for the study of 1–20 μg. dry weight. To evaluate the results for the correlation of composition with structure, an approximate estimation must be made of the relative proportion in the layer between nerve cell bodies, dendrites, threads, glia cells, and vessels. This is possible to a certain degree. The method has provided suitable material for most successful studies on the composition and enzyme activity of the nervous tissue.

The most direct and satisfactory approach with respect to cytophysiological and cytochemical work is to use isolated nerve cells or other nerve structures, provided ultramicrochemical methods permit the analysis of samples down to 10 μμg. Such methods are now available on a limited scale for the determination of chemical substances and enzyme activity. Examples will be given below. The individual nerve cell can be dissected out with a micromanipulator from 70 μ-thick sections (Edström, 1953) or by hand from thick, frozen and dried sections with sharpened needles (Lowry, 1953). The author prefers to dissect out fresh nerve cells by hand with a sharpened needle from the newly killed animal. The fresh cells can be used for all types of analyses available and have retained a large part of their dendrites (Hydén, 1959).

Methodological aspects. In some instances it has been possible to localize or quantify by cytochemical methods on sections a defined substance or activity. A cautious attitude seems, however, to be wise when staining methods are used, due to unavoidable danger of errors through diffusion, precipitation, and dissolution. If optical measurements are performed on sections to determine absolute amounts of a substance, there are two main sources of error which cannot be overlooked, especially in dealing with the nervous tissue. One is the optical inhomogeneity of the tissue irrespective of treatment (Davies and Walker, 1953; Lange, 1954; Hydén, 1955a; Rudkin and Corlette, 1957). The other is the thickness determination of a microscopic section. An accurate and rapid method for the latter purpose has only recently been developed (Hallén, 1956). Interference microscopy for mass determinations evades this difficulty and can even be used for thickness determinations, but is at present not developed technically for easy use on sections. The weight and mass for free cells are more readily accessible for determination with this method (Davies *et al.*, 1954; Davies and Wilkins, 1952; Barer, 1952; Barer *et al.*, 1953). The evaluation of the optical density over a free-lying, compressed, and highly irregular spheroid like a large nerve cell soma, however, is by no means an easy task. The result will at present be an approximate one. Two other sources of error must also be pointed out since they seriously interfere with any attempt at quantification at the cell level. The cytological procedure using chemical fixation and including embedding, cutting, and mounting, causes a high degree of volume shrinkage of the nerve tissue components. Hydén (1943b) and Brattgård *et al.* (1957) found that Carnoy's solution gives a volume shrinkage of 80 and 60 % for nerve cells from fish and rabbit, respectively. Robins *et al.* (1956) found that formaldehyde fixation causes a volume shrinkage of 80 % in rabbit material. In cases where fixed material must be used, a correction may be made, provided the fixation effect on the particular cell material has been studied.

A much more serious error than that resulting from shrinkage is also introduced by the histochemical procedure (Brattgård and Hydén, 1952). X-ray microradiography has shown that the nerve cell cytoplasm loses around 50 % of its organic material during this procedure (fixation by Carnoy's solution, embedding in paraffin, and removal from the section by benzene). The nerve cell nucleus loses 80 % or more of its material, probably owing to difference in solubility (Hydén, 1953). This is the most serious error so far encountered in histo- and cytochemical work, including electron microscopy. This type of error has also been discussed for biochemical work on cell suspensions (Allfrey *et al.*, 1955). The use of frozen and dried material is a good solution to avoid this calamity. It must be pointed out, however, that the freezing of material in liquid nitrogen, followed by embedding, is subject to the same type of error, when ethanol solutions or other solutions are used.

Quantitative relationships. The study of the composition of the nerve cell in relation to structure and function obviously requires, for many methods, information as to how large a part of the tissue is taken up by the neuron. The cell density of the cortex is discussed by several authors (von Economo and Koskinas, 1925; von Economo, 1926; Bok, 1936; Rowland and Mettler, 1949; Shariff, 1953; von Bonin, 1951; Haug, 1953; Sholl, 1953; Rebhan, 1956; Pope, 1955). To define cell density the concept of the gray cell coefficient is used, i.e., the ratio of the volume of the gray substance to the volume of the nerve cells. The term "*griseum volumen*" encompasses nerve and glia cells, vessels, and connective tissue. It seems that, passing phylogenetically upward, the nerve cells take up less and less volume than does the tissue between the nerve cells. Therefore it seems important to discuss the structural composition of the intercellular space (see below).

Based on gray-cell coefficient determinations, in man the nerve cells should occupy around 2 % of the total gray cortex volume, the dendrites, glia, fibers, and vessels, 8 %; and the intercellular space, the remaining 90 %. This seems scarcely probable. Shariff came to the conclusion that the nerve cell bodies occupy 5 % of the volume and that the average number of neurons per cubic millimeter lies near 30,000. Pope estimates that the dendrites occupy 25 %; the glia cells exclusive of the processes, 5 %; and thus the nerve cell soma plus dendrites should occupy 30 % of the cortical volume. Then 35 % remains for myelin, glia, and vessels and 35 % for the so-called extracellular fluid space. The volume of individual nerve cell body can be determined by optical measurements (Edström, 1953). The volume of the fresh nerve cell body including the dendrites can be determined by quantitative X-ray microanalysis (Hydén, 1959). The distribution of organic material, in terms of the dry weight, is fairly even in nerve cells. There is no significant difference between the nerve cell body, dendrites, and neighboring neuropile. In a frozen and dried section the mass of most structures averages 0.20–0.30 $\mu\mu g./\mu^3$. The mass of the axon, on the other hand, averages 0.06 $\mu\mu g./\mu^3$.

Extracellular fluid in relation to the neuropile. Returning to the conception "extracellular fluid space," this occupies a central position in neurobiological problems. It is a biochemically determinable factor, connected with the composition of the neuropile and related to the blood-brain barrier problem. The extracellular fluid space of the CNS has been described in analogy with the findings in other tissues. Cotlove (1932) showed that the chloride space in muscle was a measure of the total extracellular volume within that tissue. Elliott (1955) and Pappius and Elliott (1956) found that sucrose and thiocyanate do not enter the cells and could be used to measure an extracellular space of the CNS which equaled the magnitude of the chloride space. Lowry *et al.* (1954a) and Woodbury (1957) estimated the extracellular space to be around 35% of the volume of the gray matter.

The neuropile is now, by exclusion, the anatomically defined volume which can compose this space. Judged from pictures in the light microscope, it is the faintly stainable area remaining when the nerve cells, fibers, and glia are subtracted. With the electron microscope it can be seen, however, that this space is completely filled with processes of dendrites, oligodendrocytes, and the many times refolded processes of the astrocytes. These seem to project into and occupy all of the available space between the other structures of the neuropile to which also belong myelinated and unmyelinated axons. The only free spaces observable seem to be the spaces approximately 100–200 A. in width which separate the plasma membranes (Dempsey and Luse, 1958). The sum of the astrocyte processes may explain the finding of Hess (1953) that the neuropile is periodic acid-Schiff (PAS) positive. In a high-resolution study, Hess (1958) has found that the neuroglia in the cockroach is stained metachromatically.

Considering again the question where the extracellular fluid space can be located, two points can be discussed. At first sight, little space seems to be left for extracellular fluid—only a complicated system of flat channels around 100 A. deep, but with a considerable surface and no doubt providing a space for a certain amount of water solutes. If there is a 50% fluid increase in the gray matter, this would mean an increase in one dimension of only around 15%. This would be impossible to detect with present techniques in the dimension of 100 A. Therefore, it is at least hypothetically possible to explain the presence of an extracellular space in spite of the crowded appearance of the neuropile. It is more probable, however, that the notion "extracellular space" is a semantic error, adding to the anatomy of confusion. The fluid of the "extracellular space" could as well be located in the astrocytes and oligodendrocytes, the processes of which offer ample space. "Extraneuronal space" would perhaps be a better designation. Brattgård and Lindqvist (1955) have showed that bromide may be an intracellular component of the nerve cells. The data speak in favor of a similarity between bromide and chloride in this respect.

TABLE I

THE VOLUME OF NERVE CELL BODIES

Type of nerve cell	Fixed and embedded cells	Fresh cells
Bipolar cells of the retina (one large, one smaller type)	2500 μ^3 (range 1000–5000 μ^3)	—
Granular cells, cerebellum	6–700 μ^3	—
Cells of N. XII (one large, one smaller type)	5600 μ^3	13,000 μ^3
Anterior horn cells (several subgroups)	22,000 μ^3 (range 5000–40,000 μ^3)	—
Spinal ganglion cells (at least three subgroups)	—	25,000–500,000 μ^3 (median 180,000 μ^3)
Cells of nucleus supraopticus (several subgroups)	2740 μ^3 (1000–5000 μ^3)	—
Cortical cells (many subgroups)	500–20,000 μ^3	—
Cells of Deiters' nucleus		
Large type	—	90,400 μ^3
Small type	—	42,100 μ^3

If so, the measurement of chloride does not reflect only the extracellular part.

II. THE ADULT NEURON

A. *Volume, Extension, and Weight*

The volume of an adult neuron soma may vary greatly, as does the shape. Examples of the size of different types of perikaryons from the rabbit are given in Table I. It may be noted that owing to a shrinkage effect fixed and embedded cells give smaller values than fresh ones. As an example of total volume, the author has estimated the volume of a large motor nerve cell including processes to be 0.001 mm.^{3} or $10^6\mu^3$. It may also be noted that the volume of the nerve cell easily undergoes considerable changes by uptake of water or production of nucleoproteins and lipids, e.g. in nerve regeneration.

The shape of the cell body varies from almost spherical to slightly flattened prolate spheroids from which emerge the processes (Fig. 1). The ramifications of dendrites vary in number from a few to thousands and greatly increase the surface of the nerve cell. The perikaryon of a cortical cell only accounts for 10% of the total neuronal surface (Scholl, 1956). The extension of the dendrites of a Purkinje cell from the cere-

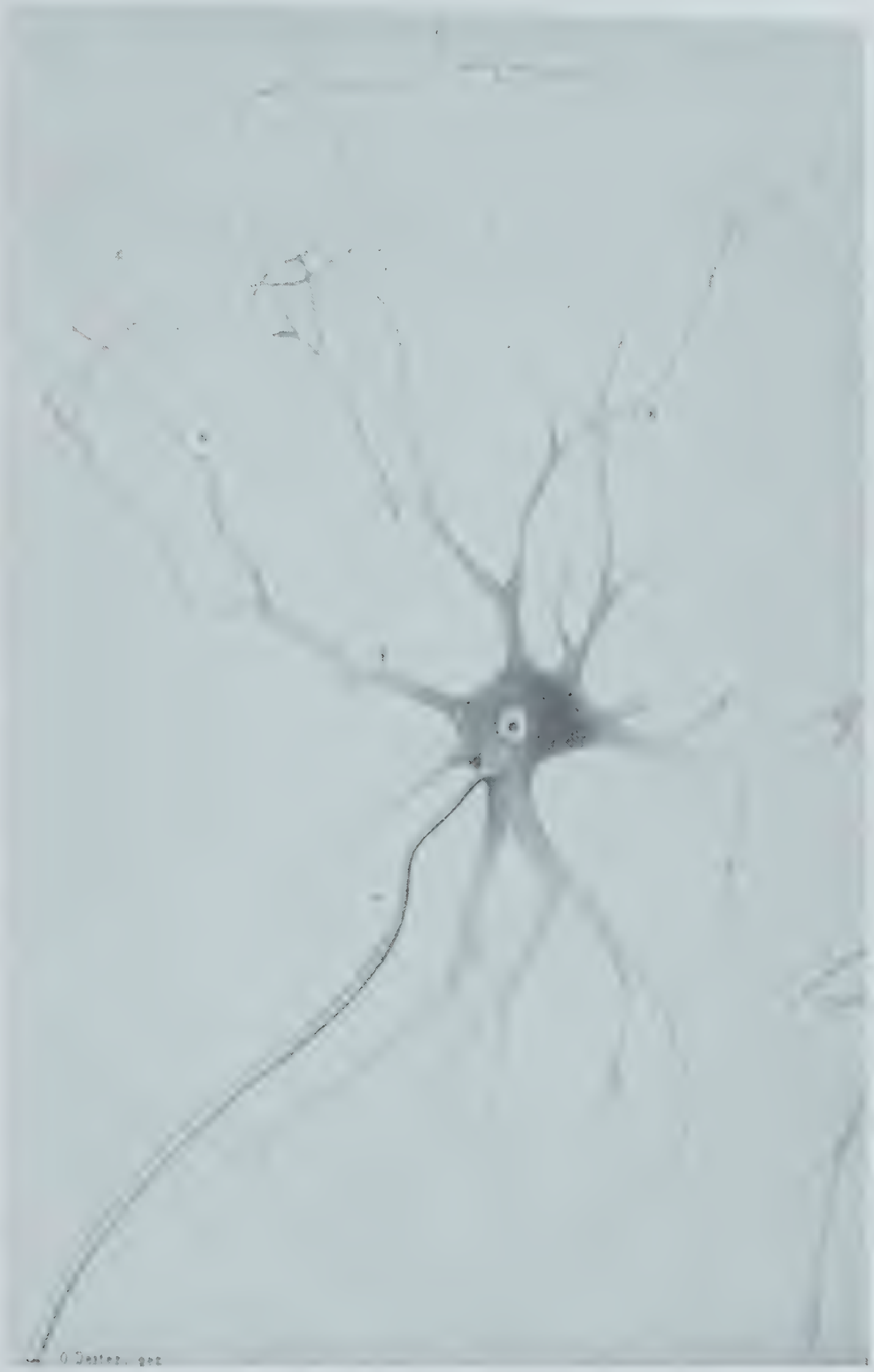
O Deiters. gez.

FIG. 1. Nerve cell from Deiters' nucleus. Dissected and drawn by Deiters (1865).

bellum is seen in Fig. 9. The surface of the dendrites of one Purkinje cell is further enlarged by the existence of small "thorns" by as much as 90,000 μ^2 (Fox and Barnard, 1957). The same authors estimated that within the ramification of the dendrites belonging to one Purkinje cell approximately 270,000 axons pass from the underlying granular cells.

Scholl (1956) estimated that within the compass of the branches of any single nerve fiber in the cortex there may be the perikaryons of 5000 neurons. The axon varies in diameter from a few hundredths of a micron to around 10 μ exclusive of the myelin sheath, and its length can amount to 1 meter. An estimate has been made that in the visual cortex the activity of one fiber may modify the space of 0.1 mm.^{3} of the cortex and its impulses may be dispersed among 5000 neurons distributed

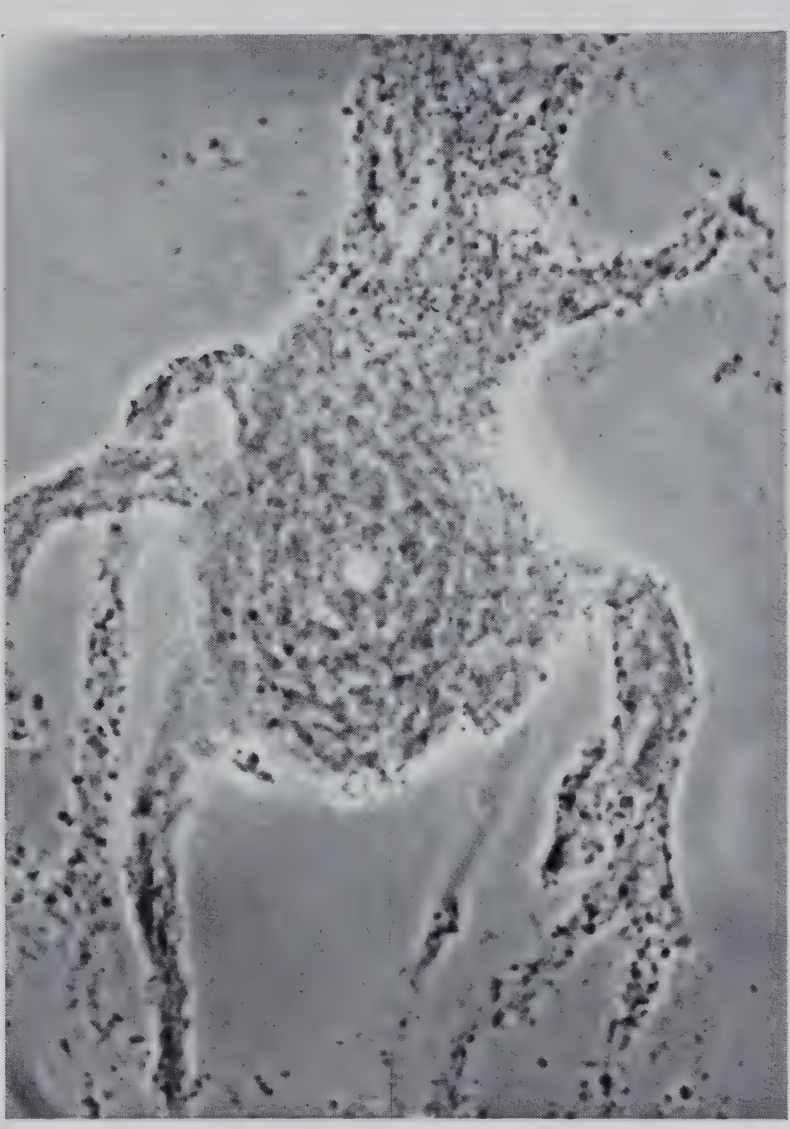

FIG. 2. Nerve cell from Deiters' nucleus. Dissected out by hand, photographed in phase contrast, in isotonic sucrose solution. Slightly stained by methylene blue to show the density of the synapses as small knobs on the surface. Magnification: × 400.

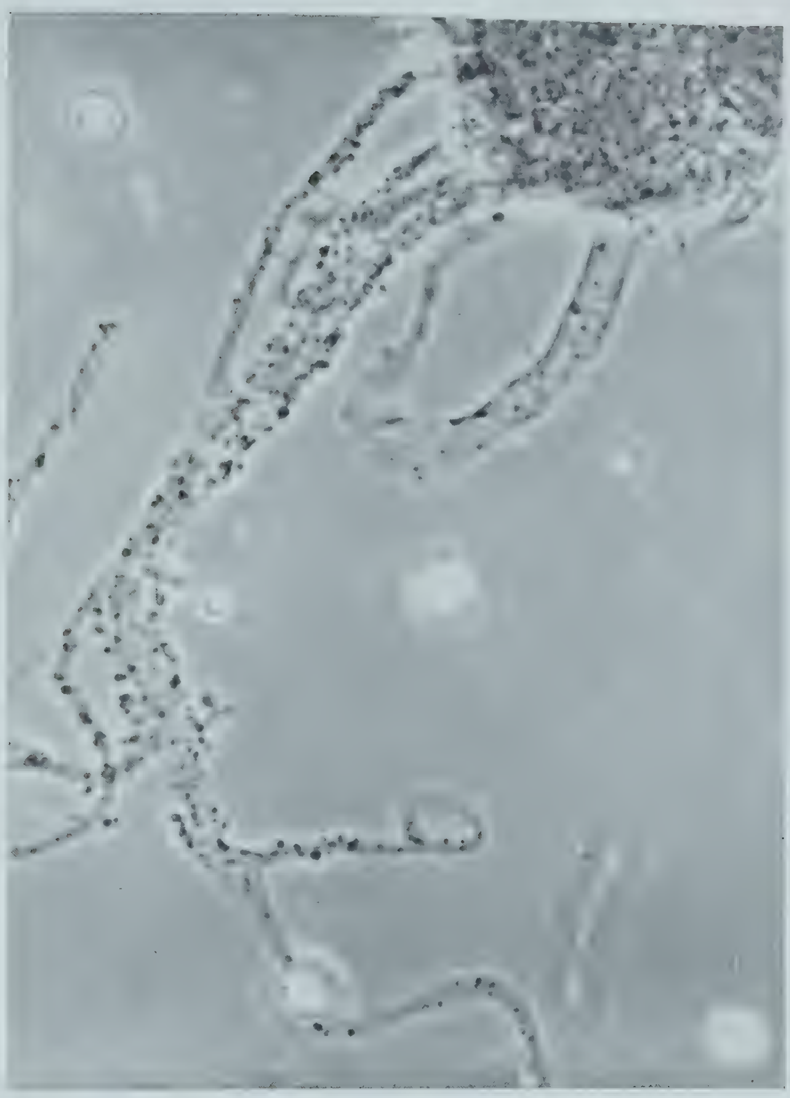

FIG. 3. Dendrites and a part of the cell body of a fresh Deiters' nerve cell, dissected out and slightly stained with methylene blue to show the number of synaptic knobs on the surface. The axon is seen to the right projecting from the body. Magnification: ×400.

around its terminal branches (Scholl, 1956). Simpler relationships are found in the motor system. To the neuron, the perikaryon, the dendrites, and the first part of the axon, attach the terminals of other neurons in the form of spherules, clubs, or more elaborately formed end-feet to a number of several thousands for cortical cells and a few thousands for motor neurons (Ramon y Cajal, 1904, 1908; Lorente de Nó, 1933). The end-feet are easily observable on the surface of a fresh nerve cell which has been slightly stained with methylene blue (Figs. 2 and 3). On many nerve cells the density of the terminals on the cell surface is high. Figure 4 shows the terminals of a myelinated nerve fiber.

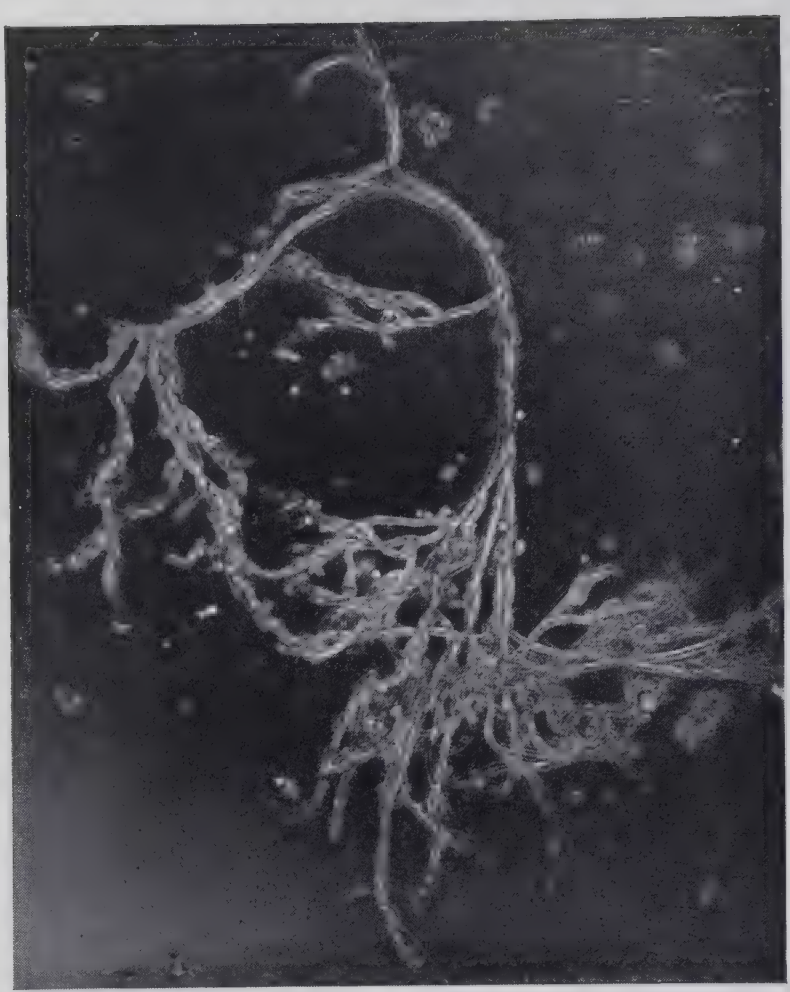

FIG. 4. Nerve fiber terminals dissected out from a fresh section through the spinal cord. Phase contrast photography. Magnification: × 150.

The neuron is the largest cell structure of the organism, highly variable in size and shape, and can be intricately formed. This unit is closely surrounded by delicate processes of the glia cells. Figures 5–9 demonstrate nerve cells exposed at 8–10 A. (as whole cells or in 5-μ thick sections) and at 2570 A.

Using a quartz fish-pole balance, Lowry (1957a) has determined that the dry *weight* of large nerve cells, spinal ganglion cells, and anterior horn cells lies in the range of 5000 to 50,000 pg.* In an example he gives average values of 10,200 pg. for spinal ganglion cells and 5800 pg. for anterior horn cells.

Using X-ray microspectrography, Brattgård *et al.* (1957) found the dry weight of hypoglossal cells to average 2770 pg. Deiters' cells, smaller type, averaged 10,550 pg. and the larger type 20,800 pg. (Hydén, 1959).

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* The abbreviation pg. (picogram $= 10^{-12}$g) has been used throughout this chapter rather than the equivalent unit micromicrogram.

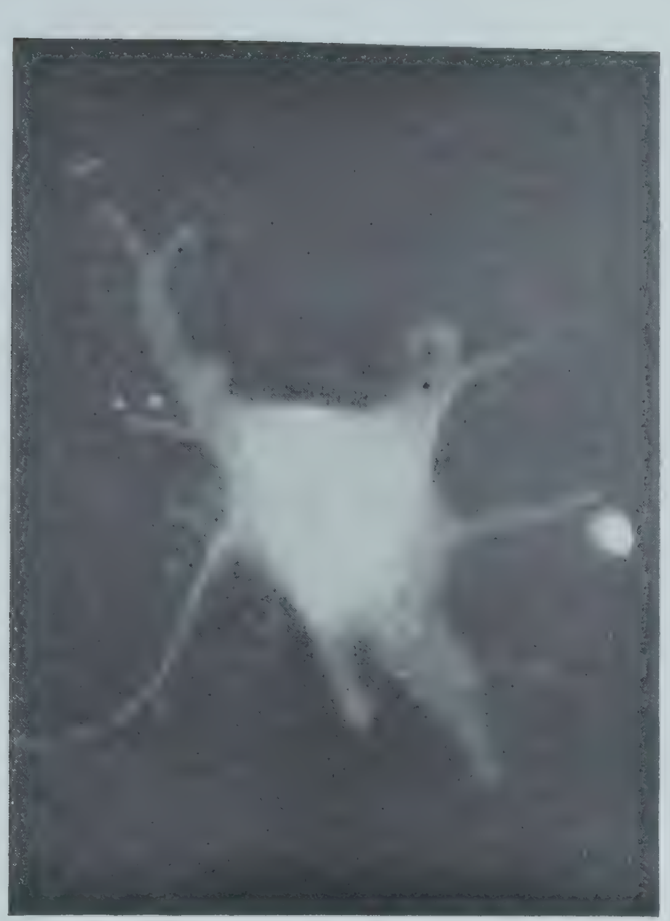


FIG. 5 a

A cell of the last-mentioned type would therefore have a wet weight of around 83,000 pg. (0.00083 mg.), assuming a density close to 1. Table II gives the weight of some types of nerve cells.

B. The Nucleus

It was early pointed out that in a given cell species there is a constant relation between the volume of the nucleus and that of the protoplasm (Hertwig, 1903). This relation seemed to be disturbed in the nerve cell. Heidenhain (1911) estimated the volume of the cytoplasm and processes to be about 125 times that of the nucleolus. He therefore considered the Nissl substance to be a nucleus-supporting material which could restore the normal nucleus-plasma relation if disturbed.

According to Bok (1936), the surface of the nerve cell body varies with the volume of the nucleus. The nuclear membrane is easy to observe in nerve cell cultures or in living, free-dissected cells. In stained preparation it appears as a thin, linear structure which in the electron micrographs appears to be a double-contoured membrane, the inner component of which is 130 A. and smooth. The outer osmophilic component is 75 A. thick and presents fine undulations and tubular extensions (Palay and

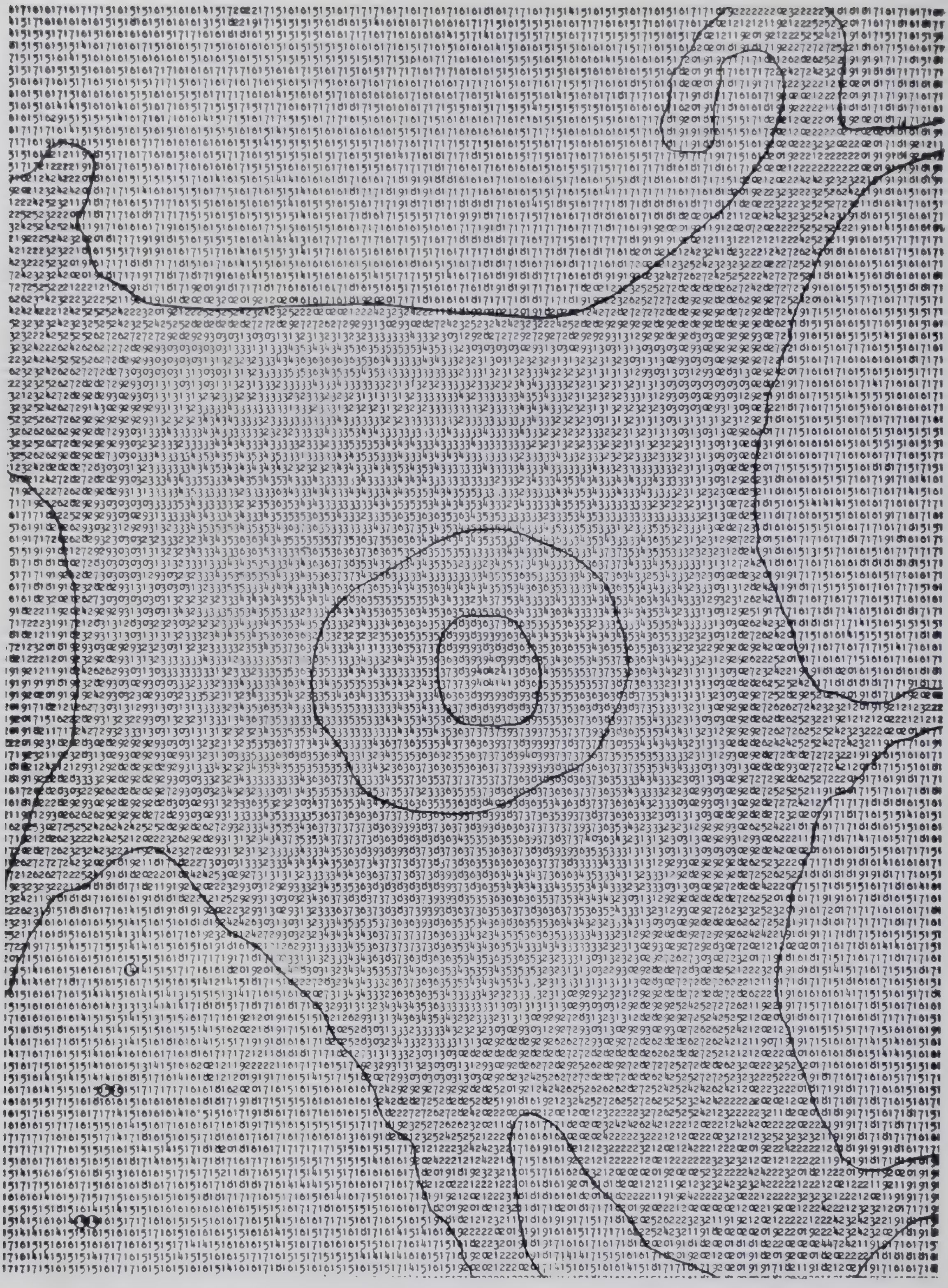


FIG. 5 b. a. Nerve cell from the Deiters' nucleus. Dissected out by hand from the fresh tissue. High-resolution X-radiograph exposed at 8–10 A. White denotes high mass. Magnification: ×3200. b. The distribution of dry organic mass within the nerve cell photographed in Fig. 5, a, as shown after the scanning and computation of the X-radiograph by the electronic computer. Two figures from 0 to 99 (see example the small rings at lower left) denote the mass in 10^{-12} gm. in an area of 3 μ^2, in this case. Approximately 700 values are computed within this nerve cell corresponding to an area of 2100 μ^2 taken up by the cell including the dendrites.

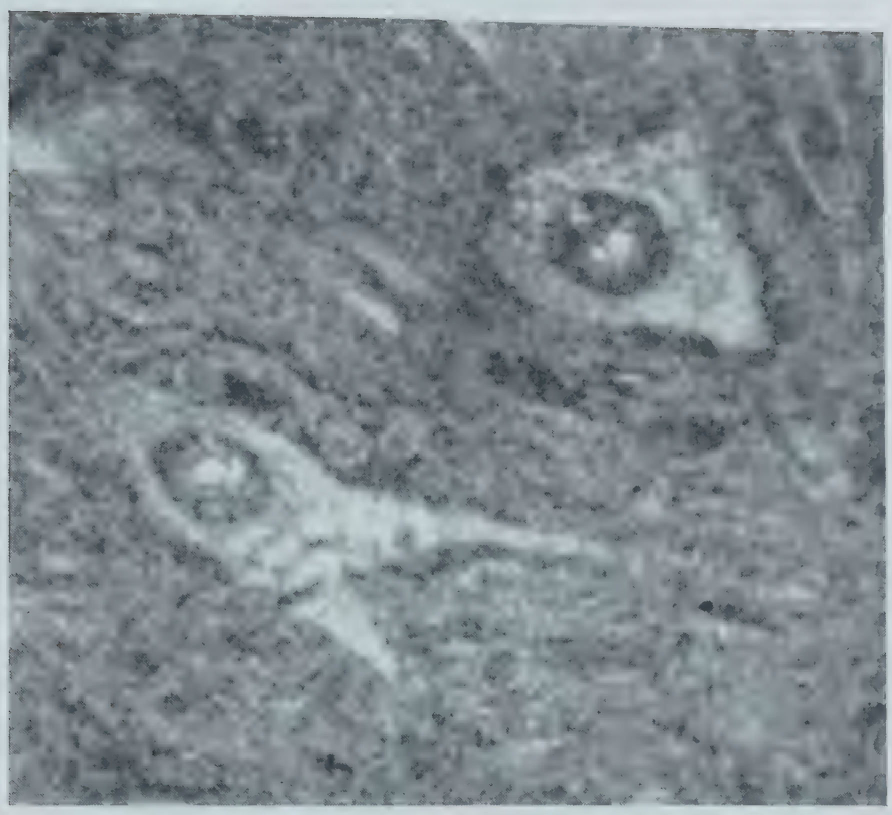


FIG. 6. High-resolution X-radiograph at 8–10 A. of a 3μ-thick section through Carnoy-fixed hypoglossal nerve cells. Structures down to 0.2μ resolved. Magnification: ×660. From Brattgård *et al.* (1957).

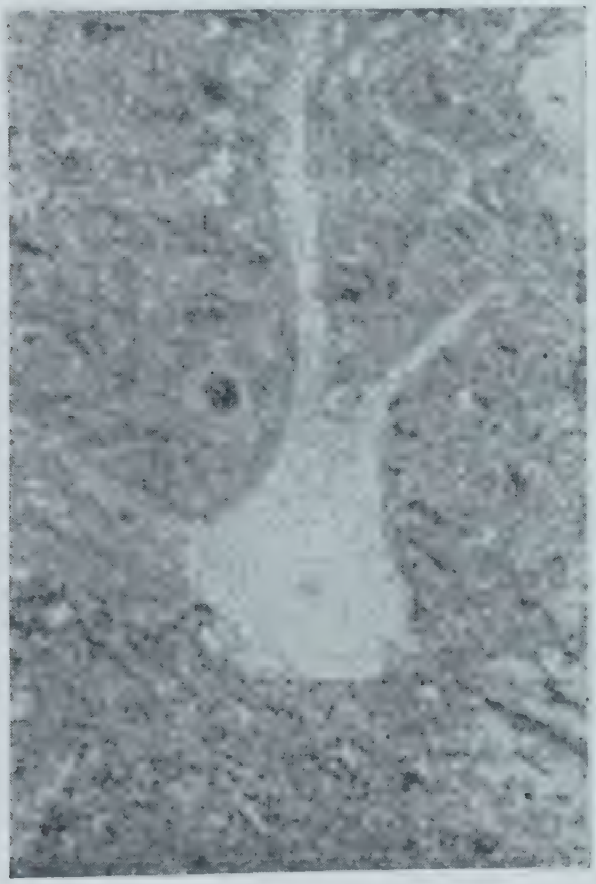


FIG. 7. High-resolution X-radiograph at 8–10 A. of a 5μ-thick section through a frozen and dried (not embedded) nerve cell from the Deiters' nucleus. Compare with cell in Fig. 6 and note that the nucleus has the same density as the cytoplasm. The reason is that the organic material still remains in the nucleus. Magnification: ×600.

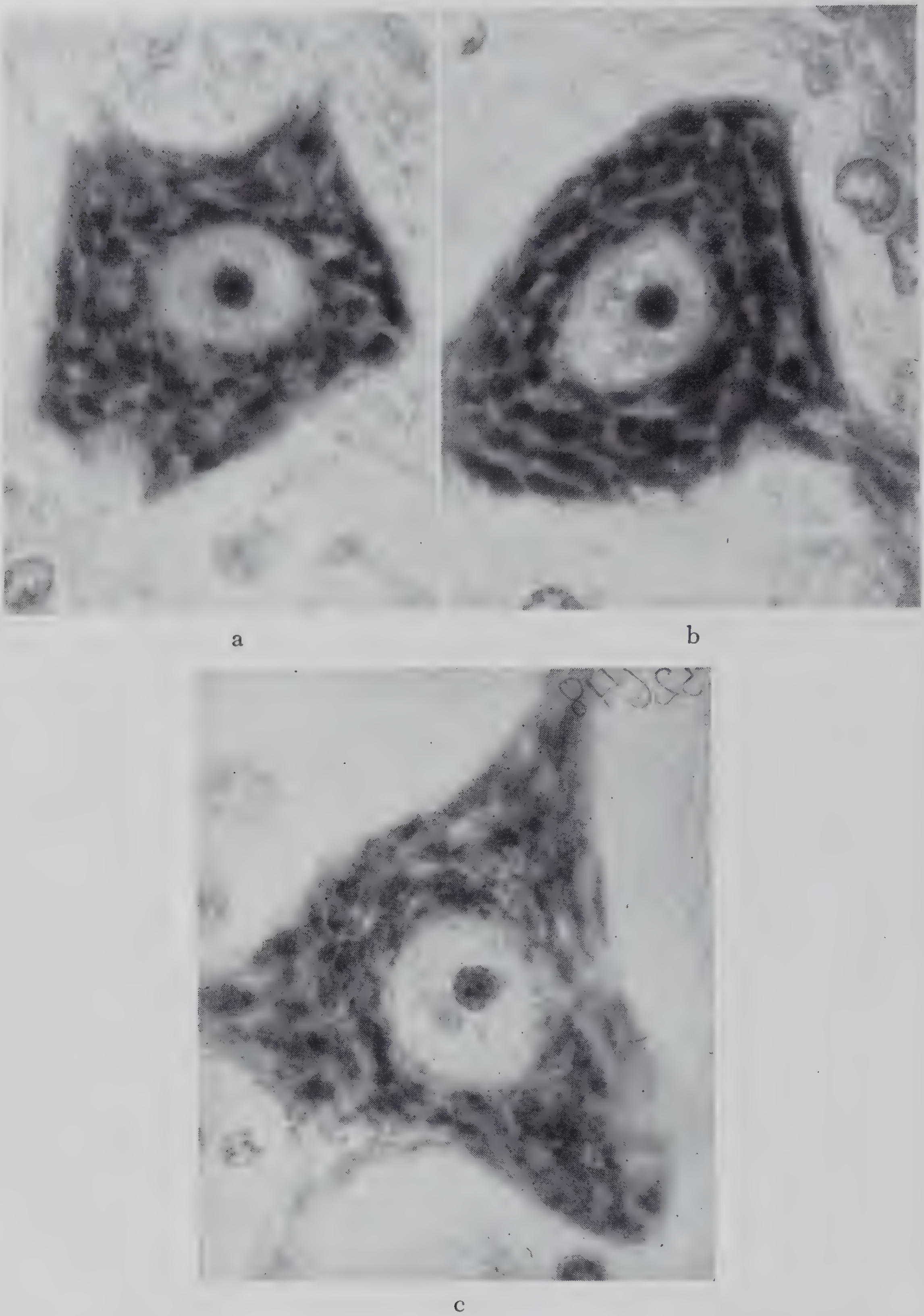


FIG. 8. a and b. Nerve cells from the Deiters' nucleus exposed at 2570 A. near the absorption maximum of the nucleic acids. c. Anterior horn cell. Note absorbing area near the nucleolus. Structures down to 0.05–0.1 μ resolved. Magnifications: ×1100 (a and b) and 875 (c). From Hamberger and Hydén (1949b).

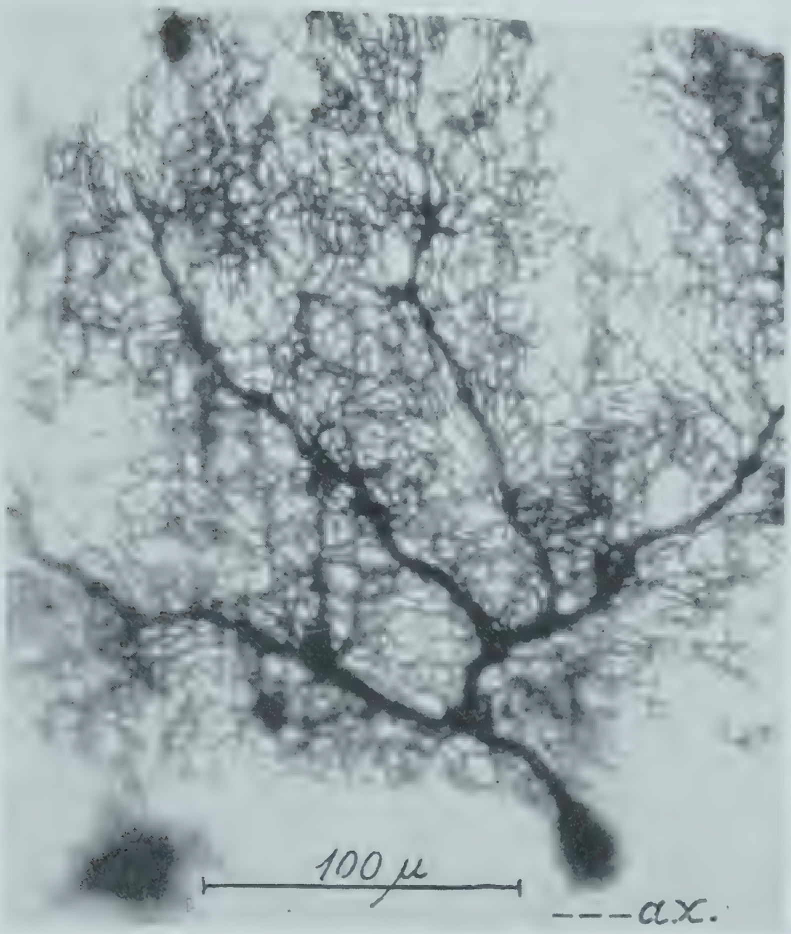


FIG. 9. Purkinje cell, stained with a modification of the Golgi technique. Courtesy of Professor A. Fox, Professor A. Brodahl, and Springer Verlag; from Brodal (1957).

TABLE II

TOTAL DRY WEIGHT OF NERVE CELLS

Cell type	Weight (pg.)[a]
Anterior horn cells[b]	9000
Anterior horn cells[c]	∼8000
Spinal ganglion cells[b]	∼10,000 (mean)
Spinal ganglion cells[c] (small to large)	5000–75,000
Hypoglossal cells[d]	2770
Deiters' cells[c]	
Small	10,550
Large	20,800

[a] Picograms.
[b] Lowry *et al.* (1956).
[c] Hydén (1959).
[d] Brattgård *et al.* (1957).

Palade, 1955; Hartmann, 1953; De Robertis, 1954a; Dawson and Wyburn, 1955). Polarization studies indicate that the nuclear membrane is composed of protein lamellae with their long axis parallel to the surface of the nucleus (Chinn, 1938). At intervals collections of dense particles around 100 A. in diameter are situated adjacent to the nuclear membrane of both sides (De Robertis, 1954b). There are also found several thin lines forming the nuclear membrane (Hartmann, 1953). The nuclear membrane of different types of nerve cells may appear folded, but even if heavily absorbing substances at 2600 A. surround the membrane on both sides it still appears as an unbroken line in the ultraviolet (UV) micrograph (Hydén, 1943a). Special interest has been focused on the submicroscopic structure of such nuclear membranes and the possible occurrence of pores in the nuclear membrane and a nucleocytoplasm, i.e., interrelationship at the macromolecular level. In pyramidal cells, nuclear protrusions are described through what seem to be gaps in the membrane, and also vesicles in the cytoplasm which communicate with the nucleoplasm (Hartmann, 1953). The author remarks, however, that the majority of data favor the view that the nerve nuclear membranes possess structural integrity. On the other hand, the occurrence of pores with or without closed edges is also claimed (Bretschneider, 1952; Dawson and Wyburn, 1955; Fernández-Morán, 1955; Palay and Palade, 1955; Roizin and Dmochowski, 1956; Horstmann and Knoop, 1957). Through these pores, exchanges or streaming of substances are supposed to occur. In summarizing the present data, and with due regard to the treatment of the nerve tissue preceding the investigation in the electron microscope, it seems appropriate to agree with Sjöstrand (1956) that the existence of holes in the nuclear membrane is not convincingly demonstrated.

C. *The Nucleolus*

In a large nerve cell, the nucleolus is a highly characteristic structure, amphophilic in its staining properties and often possessing a basophilic cortical layer, an acidophilic center, and one or more vacuoles. There are one or more nucleoli in the nerve cells even in the small types (Olszewski, 1947; Beheim-Schwarzbach, 1955). It seems not easy to give a structural, chemical, and functional definition of the nucleolus. A survey of the nucleolus problem of the nerve cell is given by Hertl (1957) and one of the problem in general by Vincent (1955). From a physiological point of view, a successively increasing body of data places the nucleolus in a central position of the cellular activity in general, especially when nucleic acid and protein production is concerned.

The volume of the nucleolus of the large-type neurons in man varies from 2 to 60 μ^3; it may be twice as large in adult specimens as in young

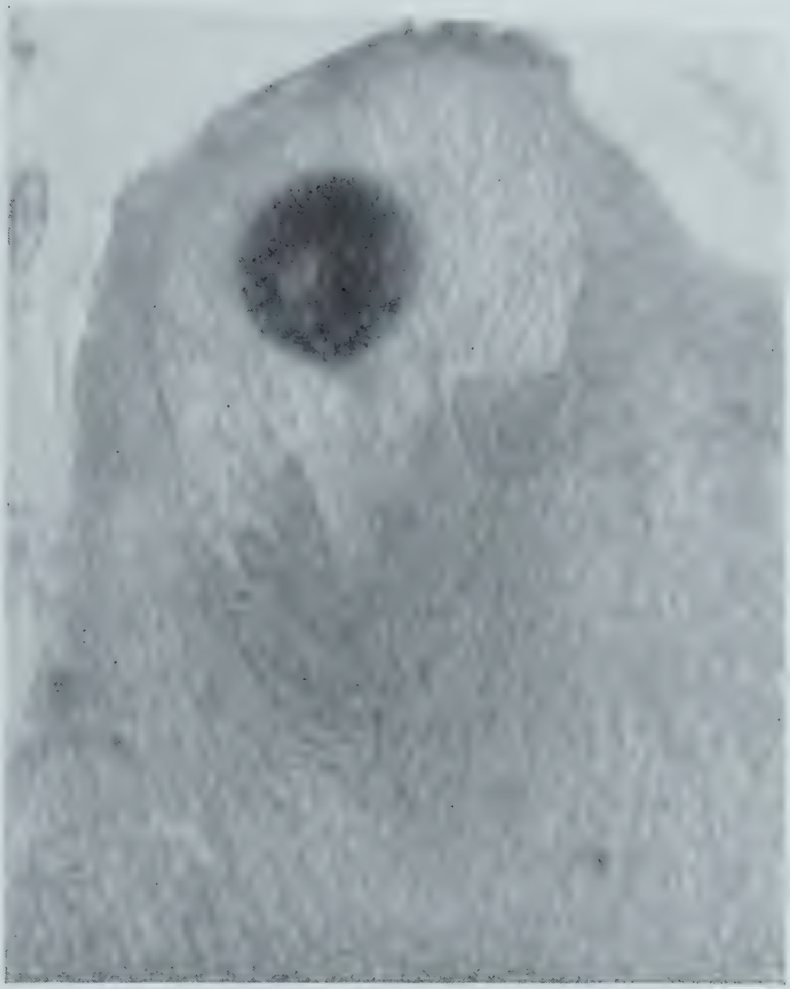


FIG. 10. Nucleus of a spinal ganglion cell of *Lophius piscatorius*, frozen and dried, photographed at 2570 A. Magnification: ×1700. From Hydén (1943).

nerve cells of the same type in mammals. In fishes there are nerve cell nucleoli of about 6000 μ^3 (Hydén, 1943b). There is no definite relation between the volume of the nucleus and that of the nucleolus. The state of functional activity seems to be a factor of importance (Grimm, 1949). In fact, motor activity can more than double the volume of the nucleoli in the motor nerve cells. This was described long ago (see also Edström, 1957). Nerve cell nucleoli may have a definite structure; Figure 10 shows a UV micrograph of a nucleolus in a spinal ganglion cell from *Lophius piscatorius*. The highest RNA concentration is distributed in a peripherally situated shell, shaped like a cogwheel in the section. In mammals most nucleoli seem structureless at an observation at 2600–3500 A. or have a few dense particles in the center. Estable and Sotelo (1951) have, however, described a threadlike structure embedded in an amorphous substance also in nerve cells. They consider this nucleolonema to be associated with the chromosomes, although Feulgen negative. It is evident that the structure of the nerve cell nucleolus may vary with type of cell and species. At high resolution Horstmann and Knoop (1957) have described a threadlike structure of granules which make up nerve cell nucleoli, but they note that nucleoli exist that lack this structure.

The nucleolus has a high mass per unit volume. Hydén and Larsson (1956) found 0.40 pg./μ^3 for fixed spinal ganglion cells, using a computer for the evaluation of the X-radiographs. Asuming a density of close to 1, this means a 40% concentration of organic material. Even higher values are reported, 50% (Brattgård and Hydén, 1952) and 70% (Nurnberger *et al.*, 1952). Since the material is lipid-extracted, the protein concentration equals the values for the mass concentration. The basic character of the nucleolar protein is shown by its high amount of free amino groups (Hydén, 1943b). All these values are found on fixed material. It is the view of the author that they must be taken with a grain of salt, since there can be expected considerable volume decrease of the nucleolus due to the cytological procedure. A fixed nucleolus has undergone such a volume shrinkage that the remaining structure can be hardly more than a small ball containing organic material in a highly concentrated form.

The RNA concentration is found to be around 0.5% in nucleoli of fish ganglion cells, 1% for mammal spinal ganglion cells, all in frozen and dried material (Hydén, 1943a, b), and 2% for anterior horn cells (Nurnberger *et al.*, 1952). These figures were the result of microspectrographic measurements. Edström and Eichner (1958b) have analyzed nucleoli from cells of the supraoptical nucleus and found 1.7 pg. RNA per nucleolus, i.e., around 20% RNA measured in weight or volume. These authors also found that the RNA content per cell body was proportional to the volume of the nucleolus (Edström and Eichner, 1958a).

Three other structures associated with the especially well developed nucleolus in the nerve cell can be mentioned. On the surface of the nucleolus appear small, DNA-rich granules or rods (Hydén, 1943b; Olszewski, 1954). By analogy with the nucleolus organizer in other organisms (plants) it has been designated "nucleolus-associated chromatin" and part of the heterochromatin. At a distance from the nucleolus are one or several areas of the same size as the nucleolus and of irregular form, which X-ray and UV analysis have shown to have a greater protein and RNA concentration than the rest of the nucleoplasm and a high amount of free basic groups. It lacks measurable amounts of DNA (Fig. 8, c). By analogy from its composition, behavior, and distribution, this substance was concluded to be a heterochromatic derivative and closely related to the nucleolus. In the male rabbit nerve cells it is more developed and frequent than in the female cells. It was therefore thought to be correlated with the Y chromosome (Hydén, 1943b). The area can increase its substance in virus infection, after stimulation, and during anoxia (Hydén, 1947, 1952a; Hochberg and Hydén, 1949; Edström, 1957). Cytologic description of this irregular area is given in the early literature (Levi, 1896; Ramon y Cajal, 1909; Saguchi, 1930). Its relation to the paranucleolar structures is discussed by Hertl (1957).

Of special interest is the occurrence of a small DNA-containing particle near the nucleolus, in its vicinity or near the nuclear membrane. It is a particle with a diameter of 0.5–1 μ, which is shown for many types of cells to be quantitatively more developed and significantly more frequent in the female cells of various species (Barr *et al.*, 1950; Barr and Bertram, 1951; Moore and Barr, 1953; Prince *et al.*, 1955). In a series of investigations this particle, the sex chromatin, has been used for sex determination with success. This sex difference also exists in neuroglia cells (Bertrand and Girard, 1957). It is thought to be a derivative of the heterochromatic regions of the X chromosomes, which combine to form the particle, thus explaining the difference in its development in the two sexes. After electric stimulation it could be seen to move from the nerve cell nucleolus toward the nuclear membrane and return again. The sex chromatin was therefore thought to be involved in protein production, like the nucleolus (Barr and Bertram, 1951).

D. The Nucleoplasm

Very little can be said at present with certainty about the structure and composition of the nucleoplasm, and the main constituent of the nucleus. One, and a most regrettable, reason is that the nucleus is the region of the nerve cell that loses most material during the usual cytological procedure. Freeze-drying followed by embedding constitutes no improved method from that point of view. The nucleoplasm in, for example, Carnoy-fixed cells has lost more than 80% of its organic material (Hydén, 1953). The old description of the nerve cell nucleus as empty, seems thus to be right. In the X-radiograph in Fig. 7 no marked difference can be seen between the nucleus and the cytoplasm in the fresh and frozen cell. Compare with the fixed and embedded cell in Fig. 6.

The mass concentration in the nucleus and in the cytoplasm of fresh, merely frozen and dried, motor nerve cells does not differ significantly and averages 0.20 pg./μ^3. It must therefore be concluded that structural studies of the nerve cell nucleus at high resolution cannot at present be expected to reflect much of the condition *in vivo*.

Interphase nuclei of oöcytes and other cells have been successfully investigated at high resolution. It seems that in such material, as well as during mitosis, the basic unit composing the chromosomes are fibrils approximately 200 A. thick (see Ris, 1957). No such systematic study has been made on the nerve cell nucleus and although granules and thin fibrils are described (Hartmann, 1953; Young, 1956; Horstmann and Knoop, 1957), it seems too early to discuss its finer structure. It is an old observation that the nerve cell nucleus seems poor in chromatin (Heidenhain, 1911). The fact that the heterochromatic chromocenters containing DNA seem scanty and far apart can be explained by a dilution effect in this large nucleus, and does not necessarily reflect a smaller content of DNA than more stainable cells contain. It can be recalled that Geitler (1939) in studies on insects found that the nerve cells remained diploid in contrast to other somatic cells during growth.

From determinations on homogenates, Heller and Elliott (1954) concluded that each nerve cell nucleus contains 7.1 pg. DNA on an average.

No reliable value of the RNA content of the nerve cell nucleus is available at present. Based on determinations on a nucleus weighing 800 pg., dry weight, and on microspectrographic measurements, the RNA content should amount to around 20 pg. It is to be noted that the RNA concentration varies greatly in different, and also in the same, types of nerve cell (Hydén, 1943b, 1947, 1950).

The nucleus has a total lipid content of 20–30%, determined chemically and by X-ray microradiography, in terms of the dry weight (Tyrell and Richter, 1951; Brattgård and Hydén, 1952). Debuch and Stammler (1956) found 5–10% lipids. Considerable amounts of cerebrosides have been found in isolated cell nuclei from the cortex. Table III is taken from the paper of Debuch and Stammler. The material was centrifuged in a sucrose

TABLE III

Composition of the Lipids of Nuclei from the Cerebral and Cerebellar Cortex (in Percentage of Total Lipids)[a]

Lipid	Cerebral cortex	Cerebellar cortex
Phosphatide:	(37.5)	(44.0)
Sphingomyelin	3.8	4.8
Lecithin	9.0	27.6
Acetalphosphatide	3.9	3.4
Cephalin	20.8	8.2
Ganglioside	1.3	0.4
Cerebroside	17.5	18.4
Cholesterin	23.0	20.5

[a] From Debuch and Stammler (1956).

solution, dried, and extracted. The concentration of dry organic substance after chloroform extraction of the fresh, freeze-dried nucleus (=protein content) lies around 20% and varies according to type of nerve cell. In general, it seems that the protein and lipid content of the nucleus is the same or just a little lower than that of the nerve cell body (Hydén and Larsson, 1956). Yet, the solubility of the substance building up the nucleus must differ considerably from that of the cytoplasm, since as much as 80% of the nuclear substance may be dissolved by the solution during a cytological or preparative procedure. Measurements on fixed nerve cell material can, however, lead to the paradoxical result of a mass concentration similar to that of the fresh material. This depends upon cell volume decrease and its effect on the sparse material left within the nucleus. In this case the distribution of the nuclear substance is highly uneven. The nerve cell nucleus, large as it is, does not lend itself easily to a closer investigation.

Richter and Hullins (1950) found in isolated nuclei from the cortex an active cytochrome oxidase, carbonic anhydrase, acetylcholineesterase, and alkaline and acid phosphatases, reflecting the metabolism of the nerve cell nucleus.

E. The Cytoplasm

The nerve cell body and the dendrites are limited by a double-contoured membrane, 100–150 A. thick (Young, 1956). So far little data are available on the structure of the nerve cell membrane and its relation to function. This is unfortunate since the membrane, especially of the neuron, plays such a central role in the discussion of the impulse and its propagation. By analogy with other cells investigated with the polarizing microscope, it may be postulated that the nerve cell membrane is composed of parallel leaflets of proteins, separated by a double layer of lipids (Schmitt *et al.*, 1935).

Especially important are the new data on the relation between the nerve soma and adjacent glia structures or fibers. The perikaryons in the CNS have generally smooth surfaces. The peripheral and spinal ganglia have folded surfaces with the thin processes projecting into the surfaces of the surrounding glia cells (Palay, 1956).

It is interesting to note that the folded nuclear membrane in nerve cells of the earthworm also shows such membrane-limited projections into the cytoplasm (De Robertis, 1954b). The same relation is evident also in ultraviolet photographs of the spinal ganglion cells of *Lophius piscatorius* (Hydén, 1943b).

Since the period 1880 to 1890, when microscope objectives sufficiently corrected for aberrations became available and aniline dyes of various types could be applied in cytology, there has accumulated a large body of literature. Every structure belonging to the neuron has its own literature: the processes, the Nissl substance, or the chromophile, basophile, or tigroid substance, the mitochondria or neurosomes, the Golgi apparatus, and (or) the Holmgren's net, the neurofibrils, the pigments, and other inclusions. For a survey and complete description, see the references on reviews. This article will attempt to discuss data relevant for the correlation of structure, composition, and function of the neuron, where such a correlation is possible—which is not often the case.

1. The Nissl Substance

In most nerve cell bodies in vertebrates and invertebrates there is present a substance, easily stainable with basic aniline dyes and in the form of large granules, thin plates, or in a more diffuse fine-granular form. It is present far out in the dendrites, but not in the area of origin of the axon, the so-called axon hillock, or in outermost periphery of the perikaryon, in a thin perinuclear zone and in the axoplasm. The substance had been described already by Key and Retzius (1876) and by Flemming (1882); it was, however, Nissl (1892, 1894a, b, c, 1896), who devised a special staining method and in a systematic study divided the nerve cells, with respect to the pattern and staining intensity of the basophile substance, into two main groups with several subgroups. Finally, he tried, in several experimental studies, to reveal its physiological importance.

In 1911 Heidenhain summarized the conception of the Nissl substance of that time. There was a correlation between the amount of the basophile substance and the volume of the neuron. He characterized the substance as a "cytochromatin" on the basis of the observations of Scott (1898) and Goldsmidt (1884) that the basophile substance emerged from the nucleus and was formed by the chromatin. It is true that Held (1895, 1897) was of the opinion that the Nissl substance, in the form in which we see it in a section, was a precipitation product and an artifact. Polemizing against this view, Heidenhain could point out that the Nissl substance in fresh cells is readily stained with methylene blue (Ramon y Cajal, 1904; Dogiel, 1897) and that it is rendered visible by the action of acetic acid solution. The Nissl substance is further visible between the area in the cell occupied by the neurofibrils and changes after stimulation of the nerve cells. All this, according to Heidenhain, indicated that the basophile substance was a vitally organized substance related to the nuclear material. Chemically the studies by Held (1895, 1897) and by Scott indicated that the Nissl substance had all the properties of a nucleoproteid that were then known. It was known that if the axons were severed, the Nissl substance underwent chromatolysis. In the subsequent changes, when the lost part eventually was substituted, the Nissl substance changed parallel with the changes in the plasma volume, thereby not upsetting the nucleus-plasma relation, and thus served a physiological purpose. The hypothesis was launched that the Nissl substance was stored in the cell during rest and was used up during the specific nervous activity. The cytochromatin should supplement the activity of the nucleus (Heidenhain, 1911).

These are all cytologically well-documented conclusions by the careful histologists of this time. How much additional data can be provided today on the structure and cytophysiology of the nerve cell?

2. *The Structure of the Nissl Substance and the Cytoplasm at High Resolution*

With the advent of electron microscopy, interest has centered on the fine structure of those parts of the neuron which had previously been discussed with respect to primary functional processes. The nuclear membrane attracts attention because of its importance in an exchange process between nucleus and cytoplasm. The Nissl substance contains a high amount of RNA and protein and is supposed to be one site of protein production, mediated by RNA and occurring at a higher rate with increased neuronal function. Besides, the Nissl substance belongs morphologically to the ergastoplasma, which is characteristic for many glandular and protein-producing cells. Moreover, it became apparent that the fine structures composing the Nissl substance were closely related to the fine system of canaliculi which crosses the cytoplasm. The mitochondria attracted evident interest because of their well-established position as a center of enzyme activity for oxidative phosphorylation. And finally, the membrane sheath of the axon and the nerve terminal are crucial structures in the propagation and transmission of the nerve impulse.

Already Nissl (1894b), Lenhossek (1895), and Lugaro (1895), had recognized the composite nature of the basophile substance and supposed an amorphous, achromatic mass which cemented together elementary, small granules which composed the basophile flakes. Ramon y Cajal (1890) and van Gehuchten (1897) considered that the basophile material stained the components of the thin framework of the ground substance. Einarson (1935) used his special staining method and found the Nissl substance to be composed of at least two components, one of high viscosity which gives rise to the typical Nissl pattern, and another, a disperse phase, with low viscosity, which forms the basophile bodies after precipitation. These examples of cytological results when the light microscope was used to the limit of its resolving power suffice. During recent years several papers have dealt with the structure of the Nissl substance as seen in the electron microscope.

Contributions have been made by Beams *et al.* (1952), Hagenau and Bernhard (1953), Wyckoff (1954), Palay and Palade (1953, 1955), De Robertis and Bennett (1954b), Fernández-Morán (1955), Ventra (1955), Dawson and Wyburn (1955), Young (1956), Roizin and Dmochowski (1956), Schultz *et al.* (1957), and other workers. The following description follows mainly that of Palay and Palade (1955).

The Nissl substance consists of the so-called endoplasmic reticulum, or, perhaps better, the cyto-membranes, a system of a three-dimensional meshwork of flat channels, tubules, and vesicles limited by membranes, and of small granules. The *first* component appears in a section as parallel, straight or curved, 60 A.-thick membranes which are regularly spaced at intervals of 800–2000 A. (Fig. 11). The lumina limited by these membranes are 300–500 A. wide with dilatations up to 2500 A. This continuous system of membrane-bound cavities is arranged in broad sheets in the areas taken up by the Nissl substance and piled one upon the other in a regular way. The cavities of this channel system anastomose with the more loosely arranged tubules and cisternae of the endoplasmic membranes in the rest of the perikaryon and the processes. In section, highly oriented profiles occur in motor neurons, but disordered ones are seen in Purkinje cells and in small nerve cells in general. Sensory and sympathetic ganglion cells represent an intermediate type.

The *second* component consists of granules, 100–300 A. in diameter, or short, dense rods. These particles are situated in rows, close to the outer surfaces of the membranes, sometimes arranged in loops, spirals, or as rosettes of five to seven granules. These granules of the Nissl substance have been thought to be responsible for the basophilia and shown to contain RNA. The Nissl substance should thus consist of dense areas in the cell body of granular-rich membranes, also designated as a reticulum. The configuration of that structure is not believed to be stable, but to change according to metabolic demands. A debate has once more arisen as to whether or not the Nissl substance exists *in vivo*. Deitch and Murray (1956) and Deitch and Moses (1957) found indications that it does. The writer's view is that a correlation has been found between the basophile substance, the RNA absorbing at 2600 A., the "endoplasmic reticulum," and the areas of higher optical density in the cytoplasm. The observations are unequivocal. But how the nucleoprotein-containing structures exist *in vivo* is quite another question.

One must ever bear in mind that these discrete particles near the membranes are observed in fixed, alcohol-treated, and embedded material. The pertinent question, therefore, is whether they really exist and have

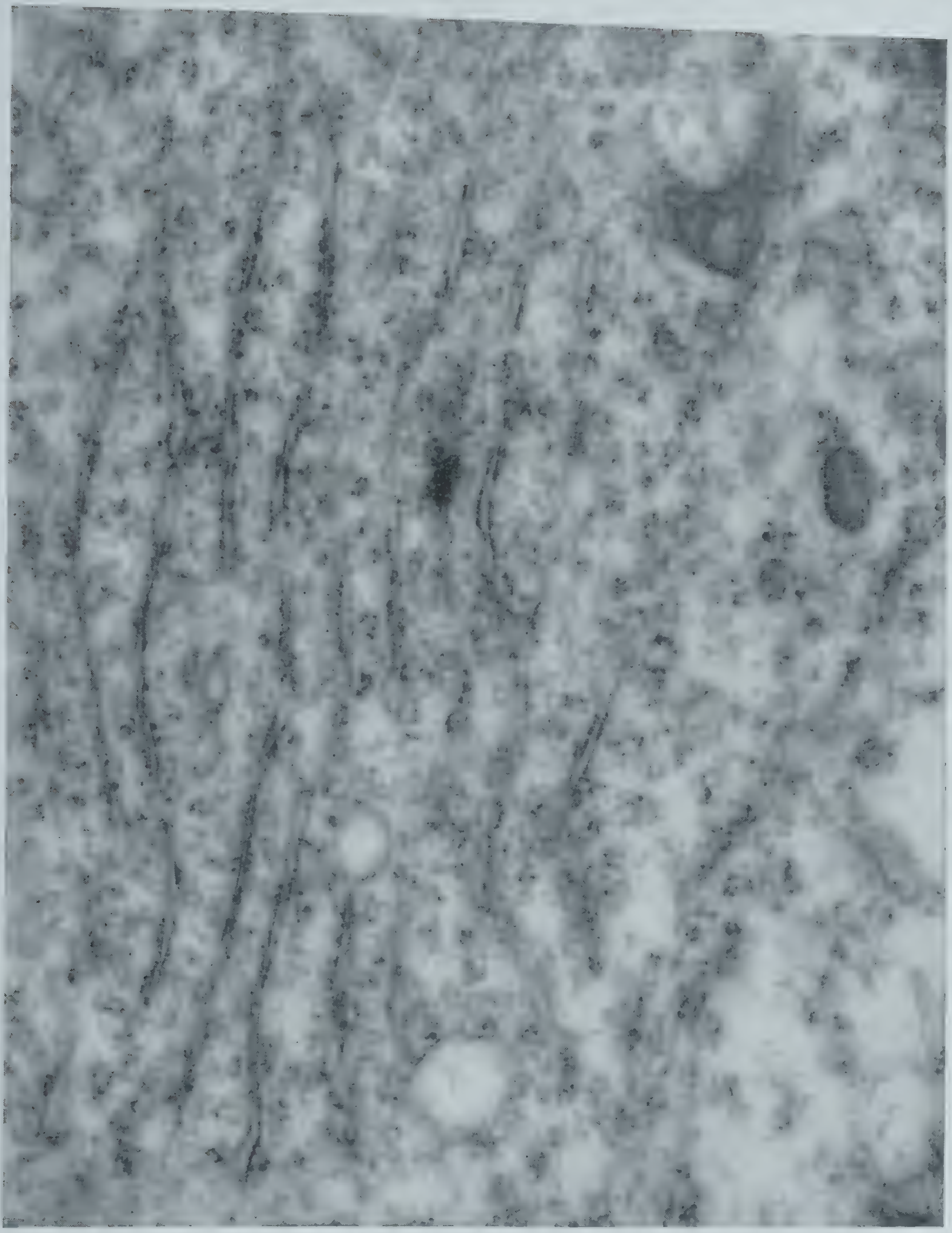


FIG. 11. Electron micrograph of the Nissl substance in a nerve cell of the abducens nucleus. Magnification: ×46,400. Courtesy of Dr. S. L. Palay.

their counterpart in the living cell. In view of existing studies on the nerve cells (see page 219), there is reason to regard with caution such small structures at such high resolution. Sjöstrand (1958) has in fact produced convincing evidence to the effect that these small particles near the membranes in glandular cells are artifacts or precipitation products caused by the cytological treatment. The Nissl substance has generally been associated with the basophilic substance, the ergastoplasm—the name coined by Garnier (1899) and Bouin and Bouin (1898)—present in other protein-producing cells, e.g., albuminous glandular cells and liver cells. The two components of the Nissl substance are found also in these cells, and the granules, the "microsomes," have been found to contain RNA (Claude, 1946; Slautterback, 1953; Porter, 1954; Palade and Siekevitz, 1956). Most of the protein and phospholipids are associated with the membranes.

3. *Chemical Composition of the Nissl Substance and the Cytoplasmic Substance*

The chemical correlate of the Nissl substance is a ribonucleoprotein probably present as *liponucleoproteins* (see Hydén, 1952a) As has already been mentioned, Held (1895, 1897) and Scott (1898) had already concluded that the Nissl substance contained nucleoproteins, but van Herwerden (1913) succeeded in removing the Nissl substance by a nuclease, Einarson (1935) also came to the conclusion that the Nissl substance contained a "nuclein" compound. Iron and phosphorus were found in the substance by Mackenzie (1897), Macallum (1898), Scott (1899), and Nicholson (1923).

The conclusive demonstration that the Nissl substance contains a ribonucleic acid with the four mononucleotides that characterize this acid was made by Edström and Hydén (1954). From dissected-out anterior horn cells the RNA was extracted and hydrolyzed from each nerve cell separately, after which the mononucleotides formed were separated by ionophoresis on a microscopic thread and identified. Edström (1956b; Edström and Eichner, 1958a) has refined this microphoretic method for the quantitative determination of purine bases and pyrimidine nucleotides in single nerve cells. It has been found that the types of nerve cells analyzed so far (Deiters', hypoglossal, trigeminal ganglion cells and neurosecretory cells from the hypothalamus) had a similar RNA-composition. The molar proportions in per cent of the sum were: adenine 19%, guanine 33%, cytosine 27% and uracil 21%. This composition did not alter during the impressive quantitative RNA changes during nerve regeneration or neurosecretion (Brattgård, Edström, Eichner, Hartmann, Hydén, unpublished).

These results confirm in principle that RNA is the substance with a high selective absorption at 2600 A. in the nerve cell cytoplasm studied with ultraviolet microspectrometry and reported in a series of papers (Landström-Hydén *et al.*, 1941, Hydén, 1950). Owing to the conjugated double bonds forming a resonance system, the purine and pyrimidine bases give the nucleic acids an absorption around fifty times that of the cell protein if the two substances occur in the same concentrations (Fig. 8). Caspersson (1936, 1940) designed a method based on these physical properties for the localization and determination of nucleic acids in cell sections or free cells. The advantage and limitation of the method has been dealt with repeatedly in the current literature (see, for example, Davies and Walker, 1953; Thorell and Ruch, 1951; Allfrey *et al.*, 1955; Hydén, 1955a; Caspersson, 1955). As a semiquantitative method the ultraviolet microspectrography clearly demonstrates gradients and differences in nucleic acid concentrations and has been of a great pioneering value. The conclusion that the Nissl substance contains ribonucleic acids was drawn by Brachet (1940) on the basis of the result of pyronine staining after digestion with ribonuclease and of the negative outcome of the Feulgen reaction and the ultraviolet spectrum reported by Landström-Hydén *et al.* (1941).

Digestion with ribonuclease and ultraviolet microspectrophotometry have also been used for better identification (Gersh and Bodian, 1943; Hamberger and Hydén, 1949a, b). Hild (1957b) made the interesting observation that ribonuclease removed the capacity of the nucleolus in the living nerve cell to bind basic dyes but left the Nissl substance unaffected. After return to normal nutrient medium for a few days, the nucleolar basophilia was restored. The reaction of nucleic acids with basic dyes has been discussed by Kelley (1939) and Michaelis (1947). The latter showed that the nucleic acids can depress polymerization of toluidine blue which then shows an absorption spectrum characteristic of the monomer of the dye. This is also the case with stained nucleic acid in histological sections.

The amount of RNA per nerve cell varies according to the type of cell. In general the values lie between 50 and 1000 pg. For the determination of such minute amounts per cell with sufficient accuracy, only one type of technique has so far proved to be satisfactory (Edström, 1953). The cell is isolated and the sample consists of cell body and the first part of the dendrites. The RNA is extracted, the extract photographed at 2570 A., and the amount determined photometrically. The method has been technically refined and has a high accuracy (Edström, 1953, 1958). If the volume is determined, the concentration in weight per volume is obtained.

Table IV gives some value obtained on different types of nerve cells,

TABLE IV

RNA CONTENT IN DIFFERENT TYPES OF NERVE CELLS

Type of nerve cell	RNA (pg./cell mean values)	Concentration (w/v)	
		Carnoy-fixed cells (%)	Fresh cells (%)
Retinal ganglion cells, rabbit[a]	—	—	—
Ganglion cells of the supraoptical nucleus, rabbit[b]	70	2.4	—
Spinal ganglion cells, rabbit[c]	1070	—	0.5
Hypoglossal cells, rabbit[d]	200	3.5	1.5
Anterior horn cells, rabbit[e]	530	2.5	—
Anterior horn cells, man[f] 40–50 years	670	—	—
Anterior horn cells, man[f] 60–70 years	540	—	—
Deiters' cells, rabbit[g]			
Large type	1550	—	1.3
Small type	700	—	1.5

[a] Edström-Eichner (unpublished).
[b] Edström-Eichner (1958b).
[c] Edström-Pigón (1958).
[d] Brattgard *et al.* (1957).
[e] Edström (1956).
[f] Hydén (unpublished).
[g] Hydén (1959).

freshly fixed in Carnoy's solution, determined in percentage of dry protein. From these values it is evident that the large nerve cell is the RNA-containing cell of choice in the organism. The only cell that can compete seems to be the pancreatic exocrine cell from small animals.

There is no difference in the quantitative result if the nerve cells are fixed directly in the Carnoy's solution or if fixation is by freezing and drying (Edström, 1956a). The concentration values, however, merit some comments. It is clearly seen how the volume shrinkage, caused by the cytological procedure, falsely increases the concentration values, e.g., in the case of the hypoglossal cells from 1.5% to 3.5%. This is of course what can be expected. Concentration values of RNA obtained with macrochemical methods on large pieces of nerve tissue are meaningless with respect to the nerve cells.

It may be of value to compare the concentration values obtained with the microchemical technique and with ultraviolet microphotometry. For material fixed according to the freeze-drying method, values of 1%

RNA in spinal ganglion cells, 0.1–2% for the different types of Purkinje cells in the cerebellum, and 2–3% in anterior horn cells are reported with the ultraviolet technique (Landström-Hydén *et al.*, 1941; Hydén, 1943a). For Carnoy-fixed anterior horn cells, 1.7% (Hydén and Hartelius, 1948; Nurnberger *et al.*, 1952), 2% for spinal ganglion cells (Hamberger and Hydén, 1945), and 1.5–2% for vestibular ganglion cells was reported (Hamberger and Hydén, 1949a, b). Summarizing, it can be said, that, irrespective of the technique used, fixed nerve cell material tends to give considerably higher RNA concentration values, owing to the error caused by the treatment, than if fresh or frozen and dried material is used.

In a study on brain microsomes, Toschi (1959) and Hanzon and Toschi (1959) found the particles of 100–175 A. in diameter, attached to the membrane structures or occurring free in the cytoplasm, to be the site of RNA. The membrane structures were found to be rich in cholinesterase.

The RNA is confined not only to the perikaryon of the nerve cell, but also to the dendrites. An approximate value of the amount of RNA in these has been obtained for Deiters' cells and lies at 100 pg., i.e., around 10% of the total amount of RNA in such a large nerve cell (Edström and Hydén, unpublished). Edström and Pigón (1958) found a proportionality between the area of the cell body surface and the content of RNA for the spinal ganglion cells. It was supposed that the membrane area was a factor determining the level of RNA but was only one of the factors influencing the nuclear size.

It is important to determine the *dry weight* of frozen and fresh nerve cells as a basis for further analyses. This has mostly been done in two ways. Lowry *et al.* (1954a, b, 1956a, b) weigh directly the dry specimen with a quartz fishpole balance. Brattgård and Hydén (1952, 1954) use X-ray microradiography under carefully controlled conditions. The results of both methods agree well. Table II gives some examples of the total dry weight of nerve cells.

In general the values lie in the range 0.15–0.25 pg./μ^3 for various cell types, higher, for example, for anterior horn cells and Deiters' cells (0.23–0.25 pg./μ^3) than for spinal ganglion cells (0.15pg./μ^3). The dry weight per volume unit has been calculated for different types of nerve cells using a scanning and computing cell analyzer (Hydén and Larsson, 1956).

As can be expected whole-brain analysis gives very little information about the protein composition of the nerve cells. The proteins of the whole brain are for the most part globulins (Demling *et al.*, 1953-1954). The amino acid composition seems to remain constant during life (Block, 1937). Histidine, however, is present in higher concentrations in adult animals than in young. There are also free amino acids, among them glutamic acid, the concentration of which exceeds that of glutamine. Using the parameters of the elementary composition of protein and X-ray spectrophotometry (Brattgård and Hydén, 1952), it has, however, been possible to compute the amount and distribution of dry *protein* in isolated nerve cells.

TABLE V

Dry Weight, Protein, and Lipid Content of Isolated Dry Deiters' Cells (Rabbit) Treated with 1 *N* Cold Perchloric Acid

Number of cells	Mean dry weight (pg.)	Volume (μ^3)	Protein (pg.)	Total lipids extr. by $CHCl_3$ (pg.)
16	10,540	42,160	8,440	2100
44	20,800	90,400	16,700	4100

As an example, there are given in Table V some values from fresh, isolated Deiters' cells, treated for 5 minutes with 1 *N* cold perchloric acid and dried in high-vacuum (Hydén, unpublished). It can be argued that the treatment with perchloric acid does not introduce an error in the weighing the cell. If anything, small amounts of glycolipids, hexose phosphates, free adenosine triphosphate (ATP) may be dissolved, but this can probably be overlooked.

The protein concentration in dry weight per volume in these nerve cells lies at 20%, and computed in terms of the dry weight, the protein concentration lies at 80%. This agrees well with the calculation of Lowry *et al.* (1954b) and also with the values of Robins *et al.* (1956). It is naturally difficult to compare results obtained with cytochemical methods with those of histochemical methods which analyze layers within, e.g., cortex. If, however, 50% of the layer is supposed to be composed of nerve cell bodies and dendrites and 50% composed of myelin-rich axons, then the values given by those two types of methods agree well.

A gradient in the cell protein has been found to extend from the nucleus to the cell periphery in Deiters' cells, amounting to around 25% expressed in percentage of the protein at the nuclear membrane (Hydén and Larsson, 1956). Fixed and embedded material gave false values due to the maltreatment.

The *lipids* make up around 65% of the dry weight of the "white matter" and 35–40% of the "gray matter" (Brante, 1949). As to the localization of the various types of lipid substances, the conclusions by inference from histochemical data are still uncertain with respect to the nerve cells.

The total amount of free lipids of the nerve cell bodies (extractable by pure chloroform from fresh dry material) in terms of the dry weight has been found to lie at 20% for many types of nerve cells (Brattgård and Hydén, 1952, 1954; Hydén, 1959). Lowry *et al.* (1954a) concluded by inference that the total lipids amounted to 2.8% on a wet weight basis, which would mean around 13% dry weight. They concluded also that the lipids consisted in equal parts of lecithin and cephalins. Lowry *et al.* (1954a) found 20% total lipids in the pyramidal layer of the Ammon's horn. Robins *et al.* (1956) found 25–31% lipids in the molecular and granular layer of the cerebellum. Nonphosphorus-containing sphingolipids (probably gangliosides) dominated in the granular layer. Brante (1949) calculated that the chief lipids of the nerve cell cytoplasm were lecithin and phosphatidyl ethanolamine in equal parts (approximately 5% of the dry weight). He also found small amounts of gangliosides, a glycolipid containing acetylsialic acid, and hexosamine. The gangliosides and their localization in the nerve cells have been more closely investigated by Klenk (1942, 1952), and by Svennerholm (1956a, b, 1957). The latter concluded that the gangliosides are localized also in the dendrites and axons. Using a staining reaction based on metachromasy and correlated with a chemical analysis, Diezel (1954a) has found that gangliosides react positively inside the nerve cells, and Brante (1955) has discussed the so-called false metachromasy given by gangliosides.

Chemically the lipids of the CNS are characterized only partially in spite of the great number of investigations. The reader is referred to recent reviews by Folch and Lees (1951), Folch (1955), Klenk (1952), Rossiter (1955), and McIlwain (1955). Still, even the classification of lipids of the CNS seems to be not more than provisional. Folch (1949) could, for example, divide the cephalin fraction into at least three phospholipids, and he made a new classification. The fractions lipoproteins and proteolipids (Folch and Lees, 1951) are probably substances functionally and structurally active within the important range 20–100 A.

At this stage of the discussion, it may be useful to show in a schematic way some chemical information as to the nerve cell at the cytoscale. Fig. 12 summarizes data obtained at this laboratory on Deiters' nerve cells of rabbit.

Return again to the question of the Nissl substance, it seems probable from the results referred to that the condensed part of the cytoplasm containing the submicroscopic system of channels and granules and giving the basophilic staining, is the remaining structure of a highly organized liponucleoprotein. In this connection is relevant the finding of Folch and Uzman (1948) of a birefringent liponucleoprotein from the CNS showing a high degree of order containing a phospholipid and a carbohydrate-rich lipid.

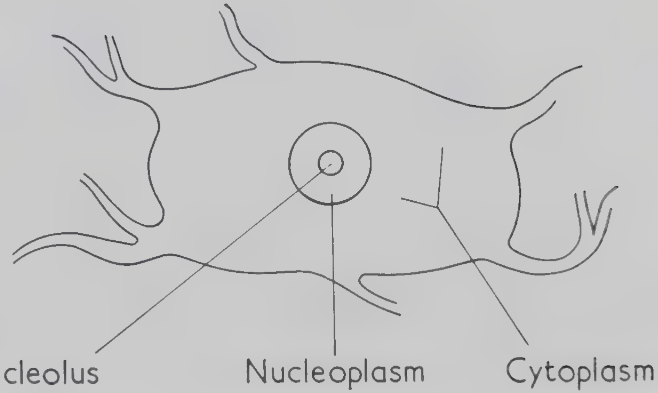


FIG. 12. Example of the chemical composition of a Deiters' nerve cell, rabbit; total dry weight, 21,700 pg.; volume, 94,000 μ^3.

Component	Nucleolus	Nucleoplasm	Cytoplasm	Dendrites	Nuclear cell membrane
Total weight/volume	100 pg.	800 pg.	20.800 pg.	—	—
Dry weight/volume	0.38 pg./μ^3	0.21 pg./μ^3	0.23 pg./μ^3	0.20–0.10 pg./μ^3	—
RNA total	—	—	1.200 pg.	~100 pg.	Concentric proteins
RNA conc. weight/volume	1.5 %	~0.5 %	1.2–1.5 %	—	
RNA conc. (% of dry protein)	—	—	7 %	—	Radial lipids (Cerebrosides)
Proteins (total)	—	650 pg.	16.600 pg.	—	
Proteins (conc., weight/volume)	—	16 %	20 %	—	—
Proteins (weight/cell weight)	—	—	75 %	—	—
Lipid fraction	—	200 pg.	4.200 pg.	—	—
Lipids (conc., weight/volume)	—	5 %	5 %	—	—

FIG. 12. Example of the chemical composition of a Deiters' nerve cell, rabbit, total dry weight 21,700 pg.; volume 94,000 μ^3.

4. Mitochondria and the Enzyme Activity of the Nerve Cell Body

The mitochondria of the nerve cell have also been called Altmann's granules, neurosomes (Held, 1897), and fuchsinophilic granules (Alzheimer, 1910). A survey of previous literature is found in a paper by Andrew (1956). Mitochondria are less numerous in the nerve cells than, for example, in intestinal epithelium or in glandular cells (Thomas, 1947, 1948; Zeiger, 1955).

The number of mitochondria vary for the different types of nerve cells. It is high in anterior horn cells, Purkinje cells, and vagal ganglion cells (Choja, 1936; Hartmann, 1948, 1949). They are mostly situated around the nucleus and between the flakes of the Nissl substance and are also present in the dendrites and the axons. They vary in size from granules 0.25–0.75 μ in diameter, to rods 0.1–1 μ long, or filaments, 2–4 μ in length (Cowdry, 1914).

At high resolution, the mitochondria are seen to be surrounded by an outer smooth membrane separated from an inner folded membrane which forms shelflike projections, cristae, into the interior (Palade, 1952). Sjöstrand (1956), interprets the pictures as showing instead an outer mitochondrial membrane and almost complete septa, inner membranes, arranged transversely or longitudinally with respect to the long axis of the mitochondrion. He could not observe any continuity between outer and inner membranes. Electron microscopy of nerve cell mitochondria is also carried out by, among others, Palay (1956), Hartmann (1953, 1956), Fernández-Morán (1955), Dawson and Wyburn (1955), and Roizin and Dmochowski (1956). In the perikaryon the mitochondrial crests are arranged transversely and in dendrites, axons, and synapses, mostly longitudinally (Fig. 13).

The double-line structure of 120–160 A. is supposed to consist of two protein membranes separated by a double layer of lipid molecules (Sjöstrand, 1953b). This interpretation now has further support (Fernández-Morán and Finean, 1957). There is no reason to believe that nerve cell mitochondria should differ in composition and function from those in other cell species. Mitochondria in general consist of 65–70 % proteins in terms of dry weight, 25–30 % lipids, mostly phospatides, and around 0.5 % RNA (see Lindberg and Ernster, 1954). These lipoprotein-rich organelles are used by the cell for the liberation of the energy of substrates and its conversion into a form suitable for the cell function. There is a concentration of respiratory and energy-transferring enzymes and coenzymes in the mitochondria. The energy liberated in the oxidations is fixed in energy-rich bonds and transferred reversibly by the mitochondria to ATP. Hereby, substances are activated for participation in synthetic processes. The liberation of energy from active mitochondria by means of

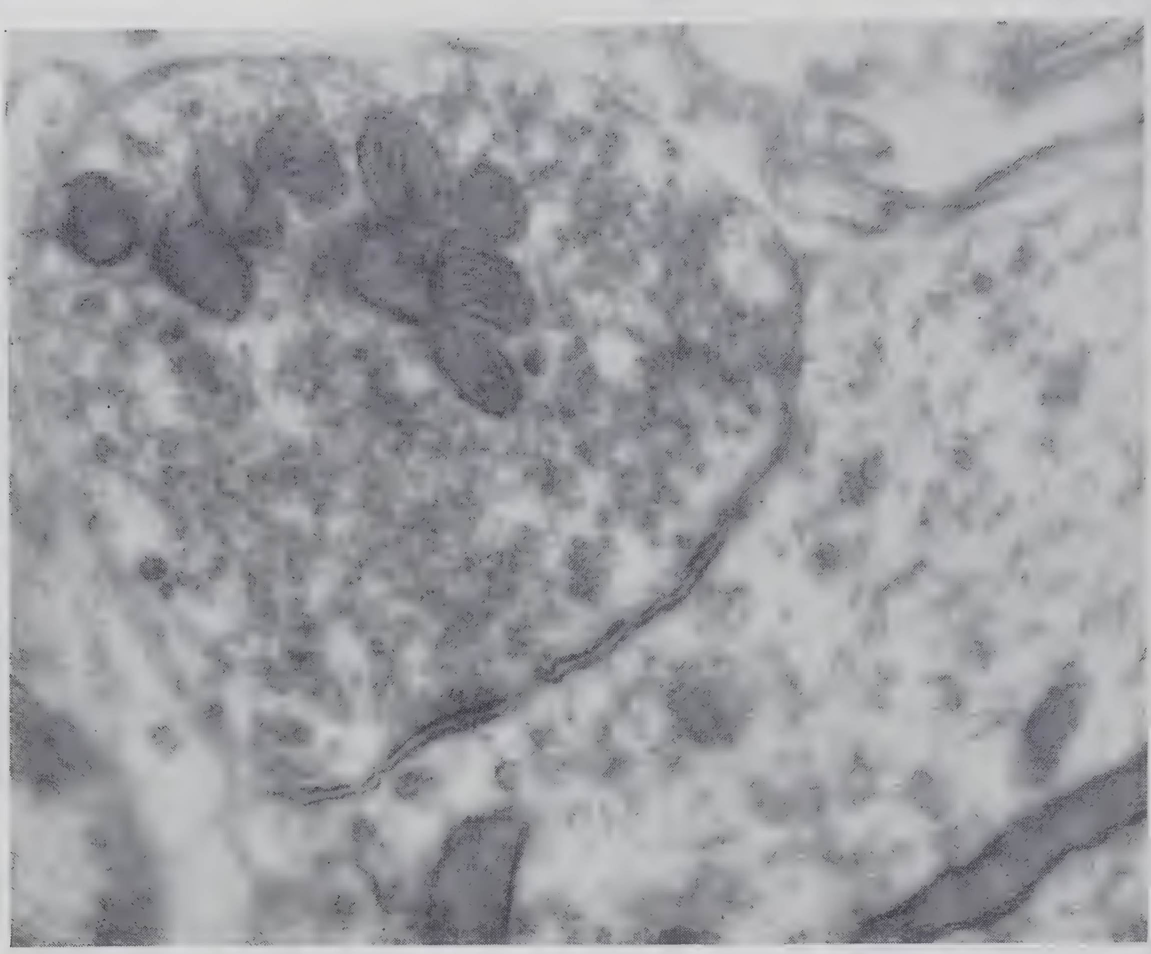


FIG. 13. Synaptic end-foot ending upon a neuron of the abducens nucleus. Magnification: ×45,800. Presynaptic area with mitochondria and vesicles at upper left, postsynaptic area to the right. Courtesy of Dr. S. L. Palay.

added enzyme systems possibly takes place at the mitochondrial surface. A gradual decrease of the mitochondrial ATP level may be reflected in a successive loss of organized enzyme properties (Ernster, 1956). The typical structure of mitochondria is supposed to provide a core and framework for a highly organized system of enzymes. Chemical results point in the same direction (Cooper *et al.*, 1955). Glycolytic enzymes and 80–90% of the cell glycolysis seem, on the other hand, to be bound to the ground cytoplasm (Lang, 1952).

Brain mitochondria, both from nerve cells and glia cells, have in general activities typical for mitochondria in other tissues. Respiratory enzymes, pyridine nucleotides, and cytochrome systems are concentrated in the mitochondria of the CNS (Brody and Bain, 1952; Abood *et al.*, 1952; Shepherd and Kalnitsky, 1954). They oxidize citric acid cycle intermediates and phosphorylates at a much higher rate than do other fractions. But they do not oxidize fatty acids, and of amino acids, only glutamic acid. The hexokinase activity which may take place at the surface of the mitochondria (Lindberg and Ernster, 1952) is relatively specific for glucose (Stein *et al.*, 1950).

In connection with the mitochondria, the localization of other enzyme activities in nerve structures may be discussed. The work referred to is mostly performed with quantitative histochemical methods. For observations obtained with specific staining reactions, see review articles by, e.g., Dixon (1954), Scholz (1957), and Feigin and Wolf (1955).

Few determinations have been made on individual nerve cells. The examples in Table VI are taken from motor and sensory nerve cells. It is surprising that the activity of the glycolytic enzyme phosphoglucoisomerase is in such great excess compared with that of the respiratory enzymes per cell.

In discussing the relative distribution of the enzyme activity within layers of the cortex Lowry *et al.* (1954a, b, 1956; Lowry, 1957b), Pope (1958, 1952a; Pope *et al.*, 1952b; Pope and Anfinsen, 1948; Pope and Hess, 1957; Pope *et al.*, 1956), and Robins (Robins and Smith, 1953; Robins *et al.*, 1956), among others, have come to the following conclusions. Respiratory enzymes, including DPN and components of the cytochrome system, are mainly localized in the mitochondria in the nerve cell bodies and dendrites. This recalls earlier observations on indophenal oxidase activity in nerve cell bodies and dendrites (Marinesco, 1919; Huszak, 1935; Roizin, 1955).

Robins *et al.* (1956) found that the glutamic dehydrogenase activity seemed to be high in the area of cortical cell dendrites but low in the Purkinje cell dendrites. Lactic dehydrogenase was high in both areas. Nerve cell bodies and dendrites seem to have the greatest capacity for glucose metabolism. Lowry *et al.* (1954a, b) and Pope *et al.* (1956) localize in general the greatest enzyme activity in the dendrites and speak of a "dendrite metabolism." The proteolytic activity reflected in peptidase activity seems also to be localized in bodies and dendrites. The acetylcholinesterase activity is mostly supposed to be localized at the surface of the axons, in and at the dendrites, and at the synapses; this is also shown by staining reactions (Koelle, 1951, 1955).

Using a microdiver technique, Giacobini (1959) could show that the cytoplasm of anterior horn cells exhibit the highest values of acetylcholinesterase activity, the axon and the dendrites slightly lower activity, and the nucleus ten to a hundred times lower activity than the other parts. The nucleolus did not show any measurable activity. He could also show that the relative acetylcholinesterase activity varied in different types of cells. It was highest in anterior horn cells, lower in sympathetic cells, and showed varying values in parasympathetic and spinal ganglion cells.

In general, in the CNS the respiratory rate seems to correspond to

TABLE VI

ENZYME ACTIVITY IN INDIVIDUAL NERVE CELLS[a]

Cell type	Enzyme	Activity (moles/kg./hr.)	Activity (μl. O_2/cell/hr. $\times 10^{-4}$)	Metabolic system
Anterior horn cell (8)[b]	Alkaline phosphates	0.08	—	Phosphoesterase
Anterior horn cell (8)[b]	Phosphoglucoisomerase	24	—	Glycolysis
Anterior horn cell (10)[f]	Glutamic-aspartic transaminase	26	—	Amino acid metabolism
Anterior horn cell (6)[f]	Glutamic dehydrogenase	2.9	—	Amino acid metabolism
Spinal ganglion cells[b]				
Large type (11)	Phosphoglucoisomerase	39	—	Glycolysis
Small type (10)	Phosphoglucoisomerase	29	—	Glycolysis
Large type (12)[c]	Succinoxidase	15	—	Cellular respiration
Large type (13)[c]	Succinic dehydrogenase	7	3.4	Cellular respiration
Large type (13)[c]	Cytochrome oxidase	20	1.6	Cellular respiration
Large type (7)[f]	Glutamic-aspartic transaminase	27.9	4.5	Cellular respiration
Small type (4)[f]	Glutamic-aspartic transaminase	31.3	—	Amino acid metabolism
Large type (6)[f]	Glutamic dehydrogenase	2	—	Amino acid metabolism
Small type (5)[f]	Glutamic dehydrogenase	3	4.4	Amino acid metabolism
Deiters' cells (13)[d]	Cytochrome oxidase	20	1.8	Cellular respiration
Deiters' cells (17)[d]	Succinoxidase	8	2–10 μl.	Cellular respiration
Symp. ganglion cell (18)[e]	Cholinesterase	9–45	(O_2/cell/hr.)	Acetylcholine turnover

[a] In terms of dry weight per living cell. The table gives for comparison the values per cell recalculated as moles per kilogram per hour based on the dry weight of these types of cells. Figures within parentheses denote number of experiments.

[b] Lowry (1957).

[c] Hydén *et al.* (1958).

[d] Hydén (1959), Hydén and Pigón (1960).

[e] Giacobini (1957), Giacobini and Holmström (1958).

[f] Lowry *et al.* (1956).

the density of the capillary net and the oxidase activity (Campbell, 1939; Meath and Pope, 1950; Opitz and Schneider, 1950; Roizin, 1955).

5. *The Golgi Net or Apparatus*

This is a structure easily demonstrated in ganglion cells by osmium or chromic silver impregnation, in the form of a relatively coarse network, "apparato reticulare" (Golgi, 1898). In 1899 Holmgren described, in the first of a series of papers, a similar structure which he maintained was the same as the Golgi net. He interpreted this structure as a system of thin channels limited by acidophile contours and produced by a liquefying of a protoplasmic network which Holmgren called trophospongium. He was of the view that this trophospongium was produced by the glia cells and carried osmophilic particles as nutrition to the nerve cells. The discussion on the Golgi net, its existence *in vivo,* and, if it does exist, its purpose, has been taken up again during recent years in connection with observations by the phase contrast and electron microscope. Gatenby (1953) claimed that he could observe the Golgi net in living sympathetic cells. It is also described in the form of osmiophilic bodies, to which have been attributed the ability to produce various substances (for a review see Hirsch, 1955). Baker (1957) is of the view that the terms Golgi net and bodies should be discarded and the structures studied in every case as cytoplasmic inclusions. This view is shared by Nath (1957).

The areas of the Golgi net or bodies are supposed to have a similar structure at high resolution as has the Nissl substance, although not associated with granules. They should consist of broad, flat channels or vesicles bounded by 100 A. thick membranes and 200–1000 A. wide, and an agranular reticulum of considerable orderliness. Lacy (1957) describes the Golgi apparatus as a chromophilic substance composed of paired membranes lying close together and enclosing a chromophobic material inside the vesicles. The paired membranes are interpreted as being delicate folds of a single such Golgi membrane. In the typical net the membranes should extend through the whole, so that the substance which the net encloses, might be regarded as being in a continuous phase.

Round or oval inclusions, 300–800 A. in diameter, which stain strongly with osmium are also found in the cytoplasm and have a cortical layer approximately 50 A. thick. They are called liposomes.

6. *The Neurofibrils.*

Neurofibrils also have a correlate in the electron microscopical picture, but their function is still unknown. The silver impregnation method of Bielschowsky shows them as a network of fine threads that appear chiefly in the areas between Nissl bodies but also through them. The neurofibrils cannot be observed in living adult vertebrate nerve cells (Marinesco and Minea, 1912; Mott, 1912; Peterfi, 1929). Thin refracting fibrils have, however, been observed in nerve cells from the jellyfish and from the lobster (Bozler, 1927; De Rényi, 1929) and in growing nerve cells (Weiss and Wang, 1936; Levi and Meyer, 1936-1937). Deitch and Murray (1956) reported that they could observe neurofibrils in living, cultivated spinal ganglion cells from the chicken. At high resolution there can be seen groups of fine filaments, 100 A. thick, that cross the nerve cell body (Palay and Palade, 1955). They concluded that if such filaments were caused to aggregate side by side by a precipitating agent and subsequently impregnated with silver, they would no doubt look like fixed neurofibrils. A general assumption is that globular proteins may form such filamentous structures by linear aggregation.

7. *Pigments and Other Constituents*

Certain types of nerve cells are characterized by the presence of pigment granules within the cytoplasm. A dark-brown melanine pigment is present in the cells of substantia nigra, locus coeruleus, ala cinerea, and in sympathetic ganglion cells. The development of the melanin occurs postnatally, in man usually within the first two years of life (Obersteiner, 1903; Mühlmann, 1900; Hueck, 1912). It is also present to a smaller extent in the nerve cells of anthropoids. Chemically, melanin from the choroid differed considerably from the composition of mitochondria, a relevant finding when the supposed production of melanine from mitochondria was considered (Stein, 1955). Melanin granules were found to have, in terms of the dry weight, $\sim$30% proteins, 1–5% lipids, $<0.3\%$ RNA, 5–10% carbohydrates, and a remarkably high content of copper, iron, and zinc. In short, few data are known about the melanin pigments in the nerve cells, their composition and function. The other type of pigment, the lipofuscin, or yellow pigment, will be discussed in Section V.

There is no *carbohydrate* reserve to speak of in the CNS. That fact, together with the high demand for oxygen supply for the maintenance of the function is remarkable. How much and which type of carbohydrates are present in the nerve cells? Unfortunately chemical and histological results disagree on this point, and both approaches seem open to criticism.

The carbohydrate content of the whole CNS in terms of wet weight constitutes around 1%; it varies for different areas and increases postnatally (see McIlwain, 1955; Schmitt, 1957a, b). Glycogen lies at 0.4–07% (Chester and Himwich, 1943). Glucose has been found in amounts of 3–6 μm./g. (see McIlwain, 1955). The best staining methods have done no more than show the occurrence of polysaccarides in the nerve cells. With the carmine method of Best, glycogen has been localized, as it has also with the tetraacetate-Schiff method (Shimizu and Kumamoto, 1952).

Polysaccarides, mucopolysaccarides, glycoproteins, glyco- and phospholipids, all can give a positive periodic acid-Schiff reaction. This reaction is rather an indication of molecular groups in the cell substance left after the cytological treatment, than a demonstration of substances.

On stimulation, adrenergic nerves, release *noradrenaline*. Its distribution and possible site of production has been dealt with by von Euler (1956), Vogt (1957), and Weil-Malherbe and Bone (1957).

5-Hydroxytryptamine or *serotonin* has been found localized more in certain parts of the brain, e.g., in the hypothalamus. It can be synthesized and metabolized by the brain. Its finer localization and function are still unknown, but all data indicate that serotonin plays an important role in the nervous function. It may be noted that serotonin occurs in large amounts outside the CNS, e.g., in the intestinal tract (Page, 1957; Udenfriend *et al.*, 1957a, b, c; Brodie and Shore, 1957).

Among *inorganic elements* the CNS contains 0.2–0.3% nonhematin iron (Tingey, 1938) and 0.4–1.5% inorganic iron. In the cortex 70% of the iron is protein bound and the relative distribution varies. Iron seems to increase postnatally beginning from very low values.

Hallgren and Sourander (1958) have determined hemin-bound iron and found that it increases in the CNS of man with increasing age. Spatz (1922) used the Turnbull blue reaction and found the intensity to vary in different areas, the iron being localized as small granules in the nerve and glia cells. Wolleman (1951) has measured photometrically the colored structures and confirmed the results of Spatz. The iron in the nerve cells has been considered to be protein bound, and Diezel (1955) has obtained ferritin crystals which he considered to be part of the physiologically occurring iron in the CNS.

The data on the copper content of the human brain vary from 0.0002% (Spatz, 1922) to 0.005 and 0.04% (see Schmidt, 1956). The copper seems not to be localized in the nerve cells, but in the glia, to judge by staining reactions (Scholz, 1957). The presence of copper in the CNS has attracted new interest since the isolation of a copper protein in three calcium fractions with a copper content of 0.3% (Porter and Folch, 1957a) and because of an increase in CNS copper content in pathological conditions (Porter and Folch, 1957b).

The *axon hillock* seems to be characterized by the same structures as the rest of the cell body: dense granules, endoplasmic reticulum, Golgi membranes, and mitochondria. It may be noted that no pronounced absorption maximum at 2600 A. has been obtained from this region.

F. The Nerve Fiber, the Axon, and the Synapses

The diameter of the axons varies considerably, from small values up to 30 μ in vertebrates, the Mauthner's fibers of fishes excluded. There is an established correlation between the diameter of the axon and the thickness of the myelin sheath (Schmitt and Bear, 1937; Taylor, 1940, 1941a, b; Werndle and Taylor, 1943; Sanders, 1948). Functionally, Gasser and Grundfest (1939) showed that there is a direct relation between the diameter of the fiber and the propagation velocity of the impulse.

What difference exists structurally between peripheral and central nerve fibers? The nodes of Ranvier and the Schwann's cells have been discussed in this connection. The peripheral myelin-rich nerve fibers are provided with nodes where the myelin sheath abruptly ceases with Schwann's cells and with a thin endoneural sheath. They are also characterized by the incisions of Schmidt-Lanterman, several to each Schwann segment, in the fixed preparations. The distance between the nodes increases with the diameter (Hursch, 1939), being 230 μ when the nodes are first laid down (Young, 1950). The distance varies in, e.g., man from 230 to more than 1000 μ. In fishes the internode length can be 8 mm.

The absolute numbers of the nodes in undivided parts of nerves were compared and a conformity was found for fibers with varying diameters, indicating a synchronism of conductivity (Thiel, 1957). The nodes are considered to represent the sole excitable parts of the axon, the internodal part serving as a conductor. According to this theory, the saltatory conduction of the nerve impulse enables an increase of the conduction velocity in the most energy-economical way (Tasaki, 1939; von Muralt, 1946; Huxley and Stämpfli, 1949). It must be emphasized, however, that the conduction velocity is dependent in the first place upon the thickness of the fiber, including the myelin sheath. Tasaki (1952) said that the theory of the saltatory conduction does not unequivocally postulate the presence of nodes. In the current literature, the term neurilemma or neurolemma denotes, on the surface of the Schwann cell, the *inner* endonerium, a network of thin argyrophile fibers (Laidlaw, 1930; Plenk, 1934). On its outer side is the sheath of Key and Retzius, the *outer* neurilemma, consisting of collagenous longitudinal fibers.

The problem has been whether the central nerve fibers also possess nodes of Ranvier and Schwann cells. For a long time it was the general concensus that they did not. On the other hand, the observations by Ramon y Cajal (1928), Bielschowsky (1928), Hess and Young (1949), Huxley and Stämpfli (1949), and Bodian (1951), to name some investigators, seem to establish that the nodes exist in the central fibers. Recently Nakai (1954) claimed to have demonstrated them after injection of osmium.

Schwann cells were supposed to be substituted by mainly oligodendroglia cells in the central fibers. Plenk (1934) claims to have evidence for the existence also in the CNS of Schwann cells, characterized by a well-developed cytoplasm. Thus, according to the papers cited, the structure of the central fibers differ from that of the peripheral fibers only in lacking the *inner* endoneurium which is replaced by the processes of the glia cells (see, however, Section II, G). According to Boycott (1903) and Takahashi (1908), the internodal parts of the peripheral fibers increase proportionally to the increase in length of the whole fiber. This seems also to be the case with the central fibers (Hess and Young, 1949). Therefore, it is better to speak of segmented myelin-rich fibers, and unsegmented, thin fibers, poor in myelin (Stoeckenius and Zeiger, 1955). Speaking against the theory of Plenk is the finding of Horstmann (1956) of direct apposition of glial cytoplasm to the myelin without any intercalated layer of a Schwann cell. The absence of Schwann cells in the CNS creates difficulties also in discussing myelin formation and segmented and nonsegmented fibers. On this point we are still confused. Both peripheral and central fibers differ in diameter, length of internodal parts, and thickness of the myelin sheath, characteristics which among others, control the velocity of the impulse propagation and the frequency.

1. Fine Structure and Composition of the Axon

A discussion of the structure and composition of the axoplasm is perhaps best started with the results of studies by polarized light (for literature references, see, among others, Bear *et al.*, 1937; Fernández-Morán, 1954; Scholz, 1957; Engström and Finean, 1958). The squid and frog axoplasm showed a relatively weak, positive form birefringence ($+1.5 \times 10^{-4}$). The analyses indicated the existence of elongated particles which were subsequently confirmed as being a fibrous protein occupying in the squid axon only a small fraction of the axon volume, about 0.07 %. Axon material from the frog was found to contain 90 A.-thick fibrils occupying approximately 0.5 volume per cent (Thornburg and De Robertis, 1956). The axoplasm exists in the form of a gel (Hodgkin and Katz, 1949a).

At high resolution the fibrous protein appears as filaments around 100 A. thick, and associated with small dense granules, perhaps indicating an axial periodicity of around 250 A. It seems probable, however, that the granular material is in a highly folded configuration or a different material (Schmitt, 1957a). In invertebrates the same thin filaments are seen, although more sparsely than in vertebrates (De Robertis and Bennett, 1955; Couteaux, 1957). The axon filaments have on the whole a parallel arrangement and pass without interruption through the nodes of Ranvier. Investigators agree that the neurofibrils in the section of silver-impregnated material represent aggregates of axon filaments side by side on which metal is deposited. Thus the bundles of filaments are large enough to be resolved in the light microscope. Fernández-Morán (1950a) describes a dense net of nodose fibrils, 100–200 A. in width, constituting the axolemma membrane. De Robertis and Sotelo (1952) and De Robertis (1954b) consider that their electron micrographs of extruded and compressed axons demonstrate the existence of an axolemma, an axon membrane, 120–140 A. thick. It is this region of the axon which is concerned when the mechanism of the maintenance of the resting potential is discussed.

The boundary between the axoplasm and the myelin sheath has been subject to particularly careful observations, e.g., by Robertson (1955a, b, 1956, 1957a, 1958). In this area is seen a double membrane, the osmophilic lines $\backsim$ 100 A. thick and the interzones 100–200 A. wide. This axon-Schwann membrane is similar in structure in both vertebrate and invertebrate nerves, and such a membrane is supposed to consist of concentric layers of protein and an interzone of a radially arranged bimolecular layer of lipids (Sjöstrand, 1953a, Finean, 1954b). The axon-Schwann membrane is continuous with a double membrane, the so-called mesaxon (see page 264), which connects with the surface of the Schwann cell. The latter also has double contours; the inner line is thicker. Robertson (1957b) maintains that each of the osmophilic lines of these membranes can be resolved in a layer of $\backsim$ 75 A. thick and consisting of two 25 A.-thick lines separated by a 25 A.-wide space. The author thinks that each of these consists of a single bimolecular leaflet of lipid with associated protein.

By X-ray diffraction technique Cohen and Geren-Uzman (1958, quoted by Schmitt, 1957a) have obtained from squid axoplasm an orientated pattern resembling the α-pattern of fibrous patterns of the KMEF class. The results indicate a definite polypeptide chain configuration of this protein.

Experiments on extruded axoplasm from the squid have supported the idea that the viscosity is caused by presence of the long protein filaments (Hodge and Jakus, quoted by Schmitt, 1957a). The protein may amount to about 2.6 % of the dry weight with the isoelectric point at pH 6.0. Maxfield (1953) showed that the axon filaments are composed of highly charged protein with a particle weight of about 10^8, that they behave in solution as flexible rods and constitute about 10 % of the protein of the nerve axoplasm. As the pH and ionic strength are increased the filaments probably decompose into globules of about 70,000 molecular weight. Maxfield and Hartley (1957) found that the axon filament diameter is greater at pH 6.0 than at pH 7.7.

The process is reversible and, according to Schmitt (1957a), may be caused by a splitting of the filaments into two components. This change may prove to be of physiological importance.

The visible change in viscosity in teased vertebrate nerve fibers was studied by Lubińska (1956a, b). The greatest structural instability was found to occur at the nodes. Deuticke *et al.* (1952) have isolated from nerves of the cow two protein components which probably represent those of the nerve fibers. The protein content of the squid axon amounts to 3–4 %, which is 30–40 % of the dry substance. The water content is over 90 % (Bear and Schmitt, 1939). These authors also isolated a nucleoprotein and a lipid complex. Edström (1956b) could not find any measurable amount of RNA in the axoplasm of the rabbit. Engström and Lüthy (1950) determined the amount of dry substance in mammal nerve axoplasm to lie at 8–9 % and Brattgård and Hydén (1952), at 6 %. Lipids constitute 33 % of the dry substance, and Brante (1949) concluded the composition to be lecithin 1.1 % wet weight, colamine-cephalin 1.1 %, cerebrosides 0.35 %, and gangliosides 0.5 %. Alsterberg (1941, 1948) supposed, on the basis of a staining reaction for unsaturated fatty acids, that lecithin and cephalin are localized in the axoplasm; this, however, is discussed by Stoeckenius and Zeiger (1955). Abood and Abul-Haj (1956) have localized unsulfonated hyaluronic acid in the axoplasm and the neurilemma sheath. In the squid the axoplasm contains no measurable amounts of cholinesterase (Boell and Nachmansohn, 1940). Specific acetylcholinesterase has been found in isolated neurites by Giacobini and Zajicek (1956), using the microdiver technique.

2. *The Synapses*

The axon process of the nerve cell enlarges in the terminal portion in various forms, thereby increasing the contact surface to the next neuron or to the effector cell. The synapse (the expression coined by Sherrington) encompasses the terminal axoplasm and the axolemma, an intersynaptic space, and the subsynaptic membrane and area. Functionally it constitutes a polarized connection between two neurons permitting a nerve impulse to pass over from one neuron to another. Observations at high resolution have unequivocally confirmed the neuron doctrine of Ramon y Cajal.

Ramon y Cajal (1909) enumerates four types of axon terminals: Small buds, end-feet, "*Endfüsse*" or "*boutons*"; irregular enlargements containing a network of neurofibrils; terminals with compact groups of neurofibrils; and, fourth, terminals in the form of small rings. They can establish an axosomatic, axodendritic, or axoaxonic contact. The size of the end-foot varies from about 0.5 to 7 μ. Bodian (1942), who has reviewed this field, has also described large monosynaptic synapses, 10 μ in diameter, in fishes. The number of terminals on large motor nerve cells may amount to around 2000 for the cell body alone and to several thousands on pyramidal cells (Lorente de Nó, 1933; Wyckoff and Young, 1954, 1956).

The end-feet vary in size on the same cell (Haggar and Barr, 1950). They can be seen on the axon hillock and on the myelin-free first part of the neurite (Bodian, 1940). There is an accumulation of mitochondria on the presynaptic side (Auerbach, 1898; Bartelmez and Hoerr, 1933; Bodian, 1940).

The electron microscope has given much information about this functionally important region. There seems to be no fundamental difference between the structure of the different types of terminals or even between those from different species. Axosomatic and axodendritic synapses have been studied by De Robertis and Bennett (1954b), De Robertis and Franchi (1954), De Robertis (1955), Engström and Wersäll (1958), Palay (1956); axoaxonic synapses, by Robertson (1955a, b), Issidorides (1957). The main findings are the following. The membrane covering the presynaptic terminal has at the site of the junction a thickness of $\sim$60 A. and appears as a single line (Fig. 13). The postsynaptic membrane has a similar appearance. The interspace is $\backsim$200 A. wide. In one or more places the presynaptic membrane exhibits increased density and thickness over a considerable area. Often the postsynaptic membrane is also thickened here (Palay, 1958). The membrane between the axoplasm and the Schwann cytoplasm appears as two 100 A.-thick lines, 200 A. apart. In the presynaptic area are seen round, denser areas, 200–650 A. in diameter. It has been concluded that these constitute spherules or vesicles covered by a $\backsim$50 A.-thick membrane. They are in most cases characteristic of the presynaptic area in the CNS and may cluster near the membrane where this appears thickened. In the hair cells of the vestibular and cochlear organ the vesicles lie in the postsynaptic part (Engström and Sjöstrand, 1954; Wersäll, 1956). It has been suggested that the vesicles may take an active part in the transmission of the nerve impulse across the synapse. The vesicles may be carriers of some thousands of molecules of acetylcholine (ACh) or any other transmitter substance. They are thought to open and empty their content into the intersynaptic space, performing a "quantal" release of ACh (see Section III, C). The indirect evidence for this suggestion is the display at individual junction regions of muscle fibers of randomly occurring pulses. These are obtained in the absence of any propagated impulse in the nerve. It was therefore reasonable to suppose that they arise from an intermittent release of small quantities of ACh bound to the vesicles. Furthermore ACh exists in a bound and a free form (Perry, 1953; Birks and McIntosh, 1957). The hypothesis is further corroborated by the finding that in synaptic degeneration there is a lysis and disappearance of the vesicles before any visible change in the membrane (De Robertis, 1955; Estable *et al.*, 1953). There is also a decrease in the ACh content of the peripheral synapse after section of the afferent nerve (McIntosh, 1938).

Synaptic vesicles have been described by De Robertis and Bennett (1954b, 1955), Palay (1954), and Palade (1954). On the other hand, Schultz *et al.* (1957) point out that such vesicles are by no means solely confined to synapses. They exist, for example, in astrocytic processes, and these authors argue that the vesicles cannot be specifically identified with synaptic transmission. By his studies of the effect of fixation solutions on the neurons, the writer shares this cautious view. It might be that the vesicles are technical products.

The presence of a dense collection of mitochondria has been confirmed in the high-resolution studies. They may lie close to the membrane. In the synapses between retinal rods and bipolar cells, there are no mitochondria (De Robertis and Franchi, 1956). Seen as a whole, the central presynaptic structure gives a denser impression owing to the presence of mitochondria and vesicles. The axon filaments or membrane-provided cisternae are absent here. The postsynaptic area does not show any other structures than those characteristic of the cytoplasm.

Many types of complicated axodendritic and axoaxonal synapses occur (Palay, 1956; Robertson, 1955b) as well as other types of synapses. These and the submicroscopic structure of the myoneural junction cannot be considered here. The reader is referred to Couteaux (1955) for a careful description of light-microscope findings and a discussion of cholinesterases at this site, and to Robertson (1957a, b), for high-resolution studies. The sarcoplasmic membrane separates the teloglia accompanying the axoplasmic projections from the sarcoplasm. The three-layered surface membrane complex which lines the invagination in which the axonal parts lie, is further complicated by secondary invaginations or folds into the sarcoplasm, $1\,\mu$ in length and 0.05–$0.15\,\mu$ in width. This constitutes in reality a thin layer of long narrow lamellae, the subneuronal apparatus (Couteaux, 1955). At high resolution, it may be seen that a multilayered membrane complex of five constituents separates the axoplasm from the sarcoplasm. On both sides is a great concentration of mitochondria and, on the presynaptic side, of vesicles. It was shown chemically (Couteaux and Nachmansohn, 1940; Couteaux, 1942) that there is a high concentration of cholinesterase in the areas of the neuromuscular junctions. Using the method of Koelle (1950, 1951, 1954), it would seem probable that a great part of the cholinesterase activity is localized at the level of the subneural apparatus (see Couteaux, 1957), at which specific receptors for ACh have been assumed to exist (del Castillo and Katz, 1956). With respect to the mechanism at the neuromuscular junction, Couteaux remarks aptly that the dominant fact is that a great part of the junction cholinesterase activity does not disappear as quickly as do the nerve endings in degeneration experiments.

G. The Myelin Sheath and the Schwann Cells

By virtue of its composition and organization, the myelin sheath has been a singularly successful subject of study with polarizing methods, X-ray diffraction technique, and the electron microscope. The details in the history of the stepwise revelation of the molecular pattern can be seen in the articles by, among others, Schmitt and Bear (1939), Edgar (1955), Fernández-Morán and Finean (1957), Scholz (1957), Schmitt and Geschwind (1957), and Engström and Finean (1958).

Ehrenberg (1849) observed that nerve fibers were birefringent. Klebs (1865) stated this was a positive uniaxial birefringence with the optic axis radially arranged. Göthlin (1913) concluded that the birefringence was due to the presence of glycerophosphatides and a crystalline structure in which cholesterol could dissolve. He divided the nerve fibers into three groups on the basis of the optic reaction. Schmidt (1924) and Schmitt and Bear (1937) carried out detailed analyses and laid the foundation for the modern conception of the structure and composition of the myelin sheath.

The studies lead to the prediction that the myelin sheath had a layered structure and was composed of bimolecular lipid leaflets, giving the positive birefringence, alternating with thin layers of protein coaxially arranged, which gave a negative birefringence. The concentric-layered structure has been found in the electron micrographs of nerve fibers fixed with osmium tetroxide, dehydrated in alcohol, and embedded in plastic. It is thus the distribution of osmophilic structures which is visualized. The repeating layer seen in the electron micrographs is about 120 A. thick, and the dense lines are ∽25 A. Frequently there is found a faint line of intermediate density, bisecting the lightly stained interbands. The spacing between the denser lines was 85–95 A. It was concluded that the 25 A.-thick lines represent the protein membranes. In some preparations it can be seen to split into two narrow lines. The distance between the principle lines corresponded roughly to the thickness of a bimolecular layer of lipids. The intermediate faint lines would then correspond to the ends of the lipid molecules in mutual contact (Sjöstrand, 1950 a, b, 1951, 1953a, b, 1954). The repeating layer in the myelin of peripheral mammalian nerves may vary from 110 A. in thin fibers to 160 A. in the thicker fibers.

Fernández-Morán (1950a, 1952, 1953b, 1954), in a series of studies, made the same observations and came to the same result. Many observers

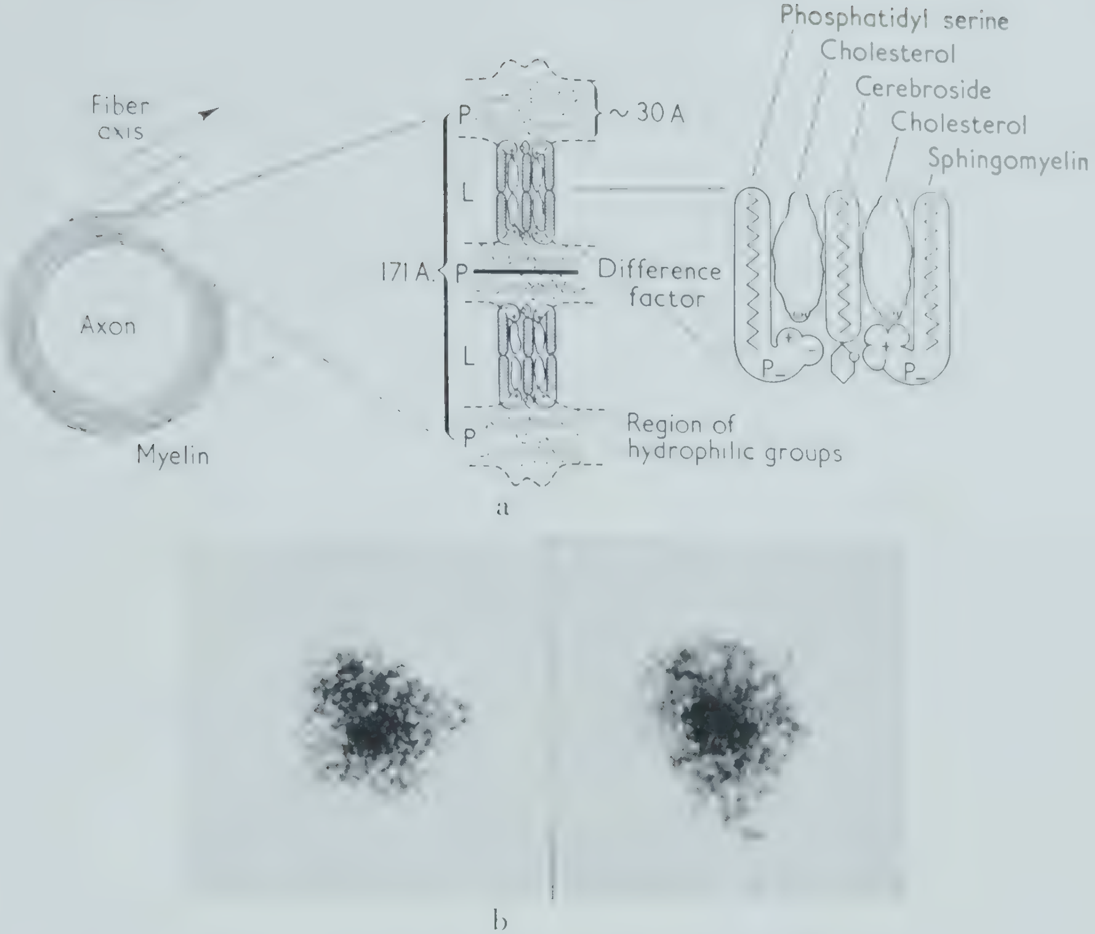

Fiber axis
Axon
Myelin
P
L
P
L
P
~30 A
171 A.
Difference factor
Region of hydrophilic groups
Phosphatidyl serine
Cholesterol
Cerebroside
Cholesterol
Sphingomyelin
P−
P−
a
b

FIG. 14. a. The molecular organization of the myelin sheath (courtesy of Dr. B. Finean). b. Autoradiograph of two regenerating hypoglossal cells, 2 hours after the injection of $P^{32}O_4$ intracisternally, showing highest radioactivity over the nucleus and indicating that the P^{32} is first incorporated into the nucleus. Magnification: ×600.

have added to this knowledge of the myelin structure (e.g., Rozsa *et al.*, 1950; Hartmann, 1951; Baud, 1952; Hess, 1953; Araki, 1956; Horstmann, 1956; Edgar, 1955).

By now it seems possible to relate the high-resolution picture to the structural arrangement obtained by X-ray diffraction data. This discussion is based also on chemical analysis of nerves. Klenk (1952) found the myelin to contain 80% lipids in percentage of the dry weight. Engström and Lüthy (1949, 1950) found a total dry weight of 30–40% and a water content of 60–70%. Brattgård and Hydén (1954) found the amount of dry substance to be around 50% in large fibers. Brante (1949) found the composition to be: cerebrosides 9.8% of the wet weight, cholesterin 8.4%, sphingophospholipids 4.8%, acetalphosphatide and serine cephalin around 4%, and sphingomyelin 2.4%.

The following description of the molecular organization of the myelin sheath follows mainly that suggested by Finean (1957) (Fig. 14). The structural arrangement in the radial direction is precise, showing a fundamental repeat period of 171 A. Within this period are further distances at 85 A. and 42.5 A. between strongly scattering groups. Furthermore, the data indicate that the 30 A.-thick line covers lipoprotein surfaces on either side of the protein layer. The intermediate line, "the difference factor," is assumed to involve ionic groups. Based on the molecular ratios 2:2:1 for the phospholipid, cholesterol, and cerebroside components of the myelin, a tentative picture of the complex structure of the lipid layer could be obtained. The phospholipids have been assumed to interact with the proteins through their phosphate groups and in a curled configuration. A cholesterol molecule has then been introduced as a stabilizing factor, forming a complex with the phospholipid molecule. The cerebroside molecule has been located between two phospholipid-cholesterol complexes. The high scattering capacity of the phosphate group is of decisive importance in relation to the relative intensitives of the X-ray reflections, and the interpretation seems to be well founded. There seems to be little free water between the lipid and protein layers. For reference, see Schmitt (1939), Schmitt *et al.* (1941), Elkes and Finean (1949, 1953), Finean (1953, 1954a, b), Fernández-Morán and Finean (1957), and Araki (1956).

That the protein layers seem to be double follows the suggested mechanism of myelin formation (Geren, B. B., 1954). According to this, the Schwann cell envelops the axon and rolls around it in a double layer. In diffraction studies the period was found to be 171 A. in amphibian nerves and 184 A. in mammals (Schmitt *et al.,* 1941). The phosphate groups are oriented toward the protein layer. The polarized end of the chain which forms a complex with the OH-group of the cholesterin is bent like a hook.

The process of myelinization in the central nervous system has been discussed by De Robertis *et al.* (1958). In high-resolution studies they have found small vesicles within the cytoplasm of oligodendrocytes closely surrounding the axons in young animals. The authors interpret the pictures as showing how concentric layers are formed from these vesicles and eventually build up the typical structure of the myelin sheath. According to this view, myelinization in the central nervous system is a process of membrane synthesis within the cytoplasm of the oligodendrocytes.

Bornstein and Murray (1958) have found an important difference between the myelinization in the peripheral and in the central fibers. The prerequisite for myelinization to start around a peripheral axon seems to be a single Schwann cell closely applied to the axon. In cultures from the central nervous tissue, Bornstein and Murray found myelinization to be limited to sites where axons were surrounded by a high concentration of neuroglia. Single contacts between glia and axon, on the other hand, did not result in formation of myelin.

There seems to be a significant difference between myelin of central fibers and that of peripheral fibers. The intermediate line of the myelin from central fibers appears to arise from groups with slight scattering capacity for X-rays, and this myelin seems to have a different type of lipoprotein association (Finean, 1953). Finean *et al.* (1957) found that peripheral nerve contains considerably less proteolipid proteins than do central nerves.

In the electron micrograph the myelin of the central fibers also shows a repeating layer of ∽120 A. but forms a structure of three closely spaced lines at the boundary of the axon. Fernández-Morán and Finean (1957) supposed this to depend upon the intermediate line, which here stains more strongly. Another less common feature, according to the same authors, is the presence around the axon of a series of layers measuring ∽60 A. In thin central fibers are still to be seen a few concentric layers rolled around the axon. Unmedullated nerve fibers have been investigated at high resolution by Gasser (1955, 1956). He found no indications of cross excitation between such axons, but a confirmation that the velocities of conduction in the fibers can be precisely accounted for by multiplying the diameters by a constant. In olfactory nerve fibers, Gasser found the small fibers to be attached to the membranes of the Schwann cells in large bundles through branched mesaxons, the fibers thus being situated outside the Schwann cell. In spite of the fact that the two types of fibers investigated have similar morphology, they differ widely with respect to physiological properties.

The Schmidt-Lanterman clefts in the myelin sheath have been shown to be shearing defects in myelin in which the lamellae are separated widely at the dense lines. The layers between the lamellae contain structures which may be components of the endoplasmic reticulum (Robertson, 1958).

The presence of nucleoproteins has been discussed by Lüthy (1946, 1951), Lüthy and von Muralt (1947), and von Muralt (1950). The latter has also localized aneurin to the myelin sheath.

The so-called neurokeratin, a precipitated and insoluble protein fraction, is supposed to exist *in vivo* in a water-soluble form in the proteins of the myelin (Block, 1938; Speakman and Townend, 1937). It is supposed to be identical with the protein part of the proteolipids (Folch and Lees, 1951). LeBaron and Folch (1956) have isolated a trypsin-resistant protein fraction containing inositol which has almost the same composition as neurokeratin and is supposed to constitute a precursor of neurokeratin. The authors assume that the classical neurokeratin is a combined degradation product of proteolipids and this combined inositol. In the fixed and stained section (e.g., with iron hematoxylin, Heidenhain, azan) the neurokeratin is supposed to constitute the delicate network of fine threads.

1. The Nodes of Ranvier

The first part of the axon is thin and devoid of myelin sheath. Then follow the segments, each consisting of neurilemma, a Schwann cell, and the myelin sheath. The ultrastructure of the nodes has been studied by Robertson (1956, 1957a) and Uzman and Nogueira-Graf (1957). The adjacent Schwann cells seem to be separated at the nodes. The gaps measure around 0.5 μ. The lamellae of the myelin sheath terminate forming an overhanging pattern and bringing each of the myelin layers in contact with the axon surface at the nodes. Robertson interprets the picture as reflecting a local unwinding of the myelin spirals. This cleavage of the myelin lamellae, if penetrated by stain, may well produce the spinous bracelet described by Nageotte. Each Schwann cytoplasm extends in tiny processes over the nodal gap.

This conclusion, if true, is of great importance, since the nodes are crucial structures as regards the bioelectrical activity of the axon. Luxoro (1956) found the Schwann cell cytoplasm to be dense in this area; this may be due to a collection of thin and contorted membranes. Thus, the nodes are not naked areas, but covered by Schwann cell cytoplasm forming a syncytium. Schmitt (1958) and Schmitt and Geschwind (1957) have discussed the implications of the various structural possibilities of the nodes as regards ion exchange. They conclude on the basis of present knowledge, that in myelinated fibers during impulse propagation, ions may pass through the inner and outer surface membranes of the Schwann cells and through their protoplasm as well as through the axolemma.

2. The Schwann Cells

These thin cells are endowed with the capacity to give rise to the myelin sheath and may be the site of high energy turnover.

Only few relevant data have been obtained so far with high-resolution studies. Most knowledge about the Schwann cell has been derived from ordinary cytological studies. The content of the Schwann cell of the squid in the electron micrograph is sparse. There are occasional mitochondria with lamellar membranes and several layers of double membranes. Robertson (1957a, b) has carefully described the double membrane structures at the Schwann cell surface. The axon surface of the cell is marked by outpocketings into the underlying axoplasm. In the latter there are often collections of mitochondria near the Schwann cell membrane.

On the basis of recent observations, Schmitt (1958) has discussed the structural relationship between axon and the satellite cells in fibers having large, intermediate, and small amounts of myelin. Various possibilities exist as regards the structure of the aqueous pathways between axon and extracellular space. In the heavily myelinated vertebrate nerve fibers, the inner layer of the Schwann cell is adherent to the axon membrane in the internodal region, possibly forming molecular contact. This leaves no aqueous channels for solute diffusion from axon membrane to extra-axonal fluid. As will be discussed below, the axon membrane seems to be covered also in the nodal regions, although more information is required before a safe judgment can be made.

The thin, vertebrate C fibers which are infolded in the Schwann cell membrane and thus in reality situated external to the Schwann cell may be in direct contact with extra-axonal fluid. They may be surrounded by an aqueous channel. Whatever the dimensions and forms of such molecular channels eventually will prove to be, the behavior of a solute in this system would be hard to predict. Furthermore, active interchange between axon and satellite cell cannot be excluded.

De Robertis and Bennett (1954a) have described 305–650 A.-large cytoplasmic vesicles interpreted as fluid globules. They suggest that the Schwann cell can mediate exchange of material between nerve cell and intercellular space. Geren and Schmitt (1955) suppose that the axon mitochondria may energize the Schwann cell interface or that the Schwann cell is actively synthesizing material which is extruded into the axoplasm.

Using empty sheaths of squid giant fibers and also their extruded axoplasm, Roberts *et al.* (1959) analyzed the activity of a number of enzymes. From the ratio of the activity of various enzymes in the sheath to that of hexokinase activity in the sheath, it seemed probable that the citric acid cycle was more prominent in the sheath cells than in the axoplasm.

The nerve cells capable of neurosecretion merit special consideration. Such cells are found in all animals which have an organized nervous system. They are especially studied in hypothalamic cells. Data indicate that the substance stainable with Gomori's method contains the hormones extractable from the neurohypophysis. Reference is made to the articles by Speidel (1919), Bargmann *et al.* (1950), and Scharrer and Scharrer (1954 a, b).

Conclusion. It is evident that the studies, especially during the last decade, dealing with the structure and composition of the neuron, have enriched our knowledge of the architectural chemistry of the neuron. Still, we have not yet approached the end of the beginning of understanding how neuronal structure integrates with composition.

III. THE CYTOPHYSIOLOGY OF THE NEURON

A. *Energy Metabolism and Synthetic Activity*

The knowledge of structural-metabolic-functional relationships in the nerve cell is scanty. It seems appropriate therefore to begin with some results of bulk analyses from which certain conclusions might be inferred.

1. *Oxygen and Glucose Utilization*

Results obtained show that the tissue of the CNS has an extremely high O_2 consumption, a rapid catabolic metabolism of glucose and of phospholipids, and a by no means slow metabolic rate of RNA and proteins. A pertinent question is, however, which structural unit is the main site of these activities, the nerve cell or the glia cells, and what is the quantitative relationship between them?

The brain uses nearly 20% of the oxygen uptake of the organism (Opitz, 1952). The O_2 consumption increases two- to fourfold after electrical stimulation. Most of the energy used by the tissue comes from the oxidation of glucose and a minor part by glycolysis. The respiratory quotient is approximately 0.8, indicating an oxidation of several substances (Brink *et al.*, 1952). The major paths of glucose metabolism in the CNS are essentially similar to those in other tissues (see McIlwain, 1955; Richter, 1955). The metabolism proceeds through pyruvate, acetylcoenzyme A, and the tricarboxylic acid cycle providing ATP or phosphocreatine, from both of which energy can be derived. It is generally recognised that the blood-brain barrier is an effective mechanism in providing a homeostasis for the CNS. As a factor introducing technical difficulties for the investigator, this barrier effect cannot be overlooked.

There has been found an inverse relation between the relative length of the internodes of embryonal nerves and the resting O_2 utilization. Later on, energy-consuming processes were more distributed along the nerve (Carpenter, 1958).

McIlwain (1953) has reported that the O_2 consumption of electrically stimulated sections through the brain is inhibited by such concentrations of narcotics, which do not depress the normal respiration of brain tissue. This indicates a difference in the metabolism of the stimulated material compared with the nonstimulated.

It has been shown that for 2 hours the O_2 consumption and electrical activity of a localized area of the cortex remained, in spite of lack of glucose, indicating that the tissue could use other substances than glucose as substrate (Grenell and Davies, 1950). Geiger, Abood, and their associates, have taken up the problem of the energy source and the functional activity of the CNS. They have found that noncarbohydrate compounds can account for the increased oxidations accompanying convulsions, and that the brain is unable to take up glucose from the blood and to oxidize if the blood has not passed the liver. Substances circulating in the blood and produced or released by the liver seem to regulate the uptake of glucose into the brain. If small amounts of cytidine and uridine were added to the perfusion fluid, the brain could be maintained in a functional condition with a normal carbohydrate metabolism even if the liver were not included in the circulation. The nature of these endogenous energy-yielding substrates are, however, so far unknown (Geiger *et al.*, 1953, 1954a,b; Geiger *et al.*, 1956; Abood and Geiger, 1955). In the perfused brain, Allweis and Magnes (1958) determined the fate of the O_2 taken up. Of the O_2 consumed, 20 % was used for the oxidation of glucose; the greater part of the remaining O_2 was found to be used for the oxidation of nonglucose endogenous substrates. The fact remains that brain can burn other substances than glucose (Gerard and Schachter, 1932; Gibbs *et al.*, 1945; see also Gerard, 1955; Richter, 1955). Of other possible substrates, glutamic acid has been discussed (Waelsch, 1955). Glutamate is able to substitute for glucose, at least in part, and maintain the oxidative metabolism of the CNS.

There exists a quantitative metabolic gradient from an intense region anteriorly in the CNS, to a less intense region posteriorly, also clearly demonstrated in the case of glutamic acid and glutamine and, no doubt, associated with a functional gradient (Gerard, 1955; Himwich, 1945). An intense activity as in convulsions or produced by electrical stimulation leads to an over-all metabolic increase. There is little knowledge about the substrate oxidized. The changes indicate an increased breakdown of the high-energy phosphate esters and increased utilization of glucose. The respiratory quotient becomes 1.0. There is an increased breakdown of creatine phosphate (Brink *et al.*, 1952). Even in states of excitement and emotional stress there are similar changes, although less in degree (Richter and Crossland, 1949).

In many such experiments, electrical pulses have been used as stimulation, (see, e.g., McIlwain, 1956). Electrical pulses were found to cause extremely rapid changes in the glucose mechanism. It is debatable, however, that electrical pulses, more or less defined, really correspond to adequate stimulation. Moreover, the author finds the notion of metabolism "at rest" somewhat inappropriate; it seems better to speak of an "active" or "more active" state of function as regards neurons and nervous tissue. Vladimirov and Rubel (1957) have pointed out that different regions in the CNS differ considerably with respect to the rate with which they exchange phosphorus compounds. Values on the macroscale are therefore hard to evaluate. The authors studied the incorporation of P^{32} into glucose monophosphate and found a decrease during hypothermia and an increase at electrical excitation.

Electrical stimulation inhibits uptake of $P^{32}O_4$ (Mullins, 1953) and inhibits also phosphorylation in frog nerves (Abood and Goldman, 1956). A passage of a current through a suspension of mitochondria also inhibited phosphorylation (Abood and Gerard, 1953). In these cases, there seems to be a real uncoupling of oxidation and phosphorylation, no doubt also the cause of the decrease in creatine phosphate. Dawson and Richter (1950) found electrical stimulation to cause a drop in ATP and phosphocreatine and an accumulation of inorganic phosphate in the brain, as did Shapot (1957). The latter also found high increase of the ADP : ATP ratio at excitation. They interpreted their results as showing how the ADP : ATP ratio determines the velocity of the energy-producing reactions in the brain.

The decrease of energy-rich phosphates at the activation of neurons seems to be due to a decreased rate of formation, rather than to an increased rate of destruction. The function of the energy-rich phosphates is not to yield extra energy for activity. A low level of such substances seems to predispose to a response, to be a characteristic of the excitable state (Gerard, 1955).

The gray of cortex has around 50% higher enzyme content and metabolic rate than does the white substance of the cord (Snyder *et al.*, 1952). As is discussed on page 250, histochemical enzyme studies have shown that there is a primary localization of respiratory enzyme activity in the mitochondria of the cell bodies and the dendrites. The activity in the dendrites seems to dominate so much that it seems fitting to speak of a "dendrite metabolism."

In an important study, Buell and associates (1958) have extended the determination of enzyme distribution in cell body layers and dendrite layers and myelinated fibers of the cortex. The enzymes were chosen so as to demonstrate the relative capacity and importance of the possible main pathways of glucose utilization.

The absolute values of the enzyme activities calculated for whole-brain tissue signified that glucose metabolism via the Embden-Meyerhof system dominated. Considering the different structure, the cell bodies had a lower content of all seven enzymes determined, whereas the dendrites were especially rich in isomerase and phosphofructokinase. There were more striking differences when the nerve cells were contrasted with the myelinated tracts. The data suggested that these are better able to use glycogen than glucose and that the direct oxidative shunt is an important pathway in the use of glucose-6-phosphate. There was a general tendency of enzymes of the same metabolic pathway to vary in constant ratio, with some exceptions. This suggested that the local concentration of an enzyme also is indicative of a metabolism along the pathway which contains that particular enzyme.

The proteolytic activity seems to be localized both in the neurons and the neuroglia (Pope, 1952a; Pope *et al.*, 1952b, 1957). The question of the relative role of the glia in these respects will be taken up in Section III, D.

2. *Lipid Metabolism*

Although formerly generally thought of as structural substances of the nervous tissue undergoing little metabolism, studies with radioactive isotypes have shown that various lipid fractions in the CNS have a considerable metabolism (see Richter, 1955; Rossiter, 1955, 1957). There is such a considerable turnover rate of brain phospholipids that an amount of phosphorus equivalent to the total amount contained in the phospholipid fraction would be exchanged in 70 hours (Dawson and Richter, 1950). Similar results have been reported by Strickland (1954) and Streicher and Gerard (1954). Dawson and Richter (1950) found a 25% decrease in the phospholipid activity ratio in the brain of mice due to emotional excitement and also a significant decrease after electrical stimulation. Geiger *et al.* (1956) found a loss of nitrogen-containing lipids from brain after electrical stimulation for only 20 seconds and suggested that phospholipids could serve as a source of energy for cerebral function. Among the phospholipids of the brain, diphosphoinositide incorporated most rapidly P^{32}-labeled phosphate (Ansell and Dohmen, 1957).

3. *RNA and Protein Metabolism*

The results of macro- and microchemical bulk analyses show unequivocally a high turnover rate of these substances in the brain and indicate a protein synthesis. On the whole, they confirm the conclusions drawn from earlier cytochemical results that there is in the nerve cells a high RNA and protein production and metabolism that increases with increasing functional demands (Hydén, 1943a, 1955a).

From the cerebrospinal fluid P^{32} is rapidly incorporated in the pentose nucleic acids (Strickland, 1952). In *in vitro* experiments this was shown to be a phenomenon depending upon the aerobic metabolism (Findlay *et al.*, 1953). The most direct studies showing a nucleic acid (and lipid) metabolism during activity are those of Geiger *et al.* (1956; Geiger, 1957). Stimulation of the brain cortex for 20 seconds was found to cause a decrease of the nucleic acids from this small cortical area, but an increase of the amino nitrogen content of the nucleic acid fraction. There was an increase of the nonprotein nitrogen content. All changes were reversible at rest after 5 minutes. They were approximately proportional to the number of stimuli. The split products from the nitrogenous components of the lipids and nucleic acids became water soluble. The authors assumed that these were bases and capable of changing the acid-base balance. By the use of specific-activity ratios Dawson and Richter (1950) showed a considerable metabolic activity in the nucleoprotein fraction of the brain. Geiger *et al.* (1956) found that stimulation for 30 seconds changed the composition of RNA in the cortex. The cytidine and adenine content of the nucleic acids increased, that of uridine and guanine remaining constant. Geiger concluded that these changes indicated a synthesis of polynucleotides, which, in turn, should induce a change in the protein synthesis. Palladin (1955), in a review on findings of Russian investigators, also emphasizes the high metabolism and a specific function of the RNA in the brain.

Isotope studies have shown that the brain protein on the whole is characterized by a high metabolism which, judged from these data, can only be assumed to be an intracellular protein metabolism. The probability seems less to favor synthetic processes for replacement of nerve tissue cells. Cohn *et al.* (1954), using S^{35}-methionine, found a higher incorporation in regions rich in nerve cell bodies than in the white substance. They could correlate this finding with a localization of the labeled amino acid to the cells. Niklas and Oehlert (1956) found also an incorporation of S^{35}-methionine into the nerve cells. Gaitonde and Richter (1955, 1956) concluded that the incorporation of S^{35} into the brain proteins is attributable to protein formation in the brain and that the free labeled amino acid, once through the blood-brain barrier, is rapidly incorporated into the protein. Lajtha *et al.* (1957a), Waelsch (1957), and Lajtha *et al.* (1957a, b) concluded on the basis of isotope studies using S^{35}-lysine that there is most likely a dynamic state of the brain proteins. There are proteins with a high and a low turnover rate, as can be expected in such an inhomogeneous tissue. The shortest value for the half-life of the protein was 2.2 days for the whole brain. The highest rate of incorporation was found in the corpus callosum. A comment here would be that the glia cells might be suspected to account for the result. The authors found further that the microsomal fractions incorporated at the highest rate. Half the free lysine was replaced in 45 minutes, and the authors discuss the possibility that the movements of lysine and potassium may be interdependent. In this connection, the finding of basic, water-soluble split products of lipids and nucleic acids after stimulation is relevant Geiger *et al.*, 1956). After stimulation, Ungar *et al.* (1956a, b) found indication of a reversible change in the configuration of protein in the direction of a denaturation. Prolonged stimulation caused a breakdown of proteins and the appearance of proteolytic activity. On the other hand, Abood *et al.* (1958) found a significant decrease of free amino acids, using, however, frog nerves after stimulation. They found no decrease in any of the nucleotide components, but an inhibition in the turnover of the phospholipids and in a phosphopeptide complex. Glutamic acid is an important factor in the transamination system and in the glutamic dehydrogenase system and may bind free ammonia, which is liberated at increased activity and must be promptly removed (see further Richter, 1955; and Waelsch, 1955).

Ungar *et al.* (1956a; see also Winterstein, 1929) studied the nitrogen production, especially the formation of ammonia, using frog spinal cord, and found it to increase approximately threefold after electrical stimulation. Gerard (1955) found this liberation of ammonia to be threefold greater than reported, since two-thirds is retained by combination with glutamic acid. Weil-Malherbe and Green (1955) suggested that the formation of ammonia was linked with proteolysis. Vrba (1955a, b, 1956) studied glutamic acid changes in the brain after intense motor activity and found a decrease of the free glutamic acid and the Kjeldahl nitrogen concentration of the protein fraction, formation of ammonia which was bound as glutamine, and consequently an increase of free glutamine. As an indication of degradation of nitrogen-containing components in the proteins during activity, a decreased concentration of amide groups was found. The author concluded that there is an increased protein metabolism in the brain during activity and that the free glutamine serves also as a basis for resynthetic processes.

In a later paper, Vrba and Folbergrova (1959) described the fractionation of nitrogen compounds in fresh and incubated brain cortex slices. The decrease of the nitrogen in the protein and nucleoprotein fractions during incubation could be accounted for by an increase of ammonia of nonprotein nitrogen and of lipid nitrogen. They assumed that these degradation processes were accompanied by utilization of endogenous bound pentose.

Summary. Some highly important data for the further discussion of metabolism at the neurocytoscale have arisen from the macrochemical studies cited above. Lipids, RNA, and proteins of the CNS are characterized by a high metabolism, which is further increased at increasing activity when a synthesis of these substances occurs. It is interesting to note that substances, previously regarded as "structure-forming" materials, do have a considerable metabolism in the CNS. At a certain point in increased neuronal activity, there occurs a shift in the substrate for the metabolism which differs from that of a less active state. This phenomenon has not so far been clarified. The studies cited cannot, however, tell the site within the nervous tissue of these various activities and energetic processes. Are the substances metabolized in the nerve cells, their dendrites, neurites, or in the glia cells which dominate numerically? Is it a nutrient metabolism or an expression of a specific functional synthesis, and if so, which is the intraneuronal mechanism for the synthesis?

4. *Changes Indicative of Intraneuronal Nucleoprotein Production and High Metabolism Linked with Neuronal Activity*

Recent studies of nerve cells during regeneration have given results of interest for the RNA and protein production in nerve cells in general (see page 295). After crushing of a nerve, new thin axons begin to grow out almost immediately, and with a high velocity. During this stage the cells are increased in volume and show chromatolysis. When the contact with the effector organ is reached, the new axons continue to grow in thickness. The chromatolysis persists for a considerable time. During the first of these stages Brattgård *et al.* (1957) found a doubling of an increase in the amount of proteins and lipids in hypoglossal nerve cells in spite of a concentration decrease. The amount of RNA per cell did not at first increase, but the state of aggregation changed toward smaller particles. This was interpreted as a change from an active to a more active state. Using C^{14}-orotic acid, Brattgård *et al.* (1958) found that the chromatolytic regenerating nerve cells at this stage incorporated considerably more of the orotic acid than did the control cells; this indicated an increased turnover rate of RNA. During the continued regeneration, the amount of RNA per cell increased tremendously, as did the proteins and lipids. Fischer *et al.* (1958) found that regenerating nerve cells incorporated more S^{35}-methionine per unit area than the control cells, indicating an increased protein synthesis. The examples cited show an increased *synthetic metabolism* which can be correlated with the retrograde cytological changes of the nerve cells.

Using C^{14}-adenine, C^{14}-orotic acid, Koenig (1958) found radioactivity over the nucleus already 4 hours after the injection. The blackening gradually increased over the cytoplasm with the exception of the axon hillock and still remained 50 days after the injection. The specificity of the blackening was controlled with ribonuclease and deoxyribonuclease. When S-labeled methionine was used, the radioactivity over the nerve cells could be shown within 1 hour and began to decrease 4 hours after the injection. The author concludes that a rapid turnover takes place in the nerve cells of ribonucleoproteins.

These and other recent investigations performed with accurate quantitative methods now available contribute more support to the conclusions drawn from results of a series of studies with UV microspectrography by the writer and associates (1941–1957). These results indicated a production of RNA, proteins, and lipids in the nerve cell accompanying increased neuronal activity. Adequate stimulation for a short time may increase the concentration of RNA in proteins. Prolonged or very intense stimulation may lead to a reversible decrease in the amount of these substances. Large differences in the RNA, proteins, and lipids of the individual nerve cells may reflect differences in the functional state. RNA concentration changes, occur rapidly in the nerve cells with increasing functional demands, no doubt following rapid volume changes.

A technical comment may be made in connection with determinations with UV microspectrography; it does not affect any conclusion made on the basis of such results, but concerns the absolute values of nucleic acids inferred from the absorption spectra. When a cell undergoes rapid volume changes the amount of nucleic acids may well increase although absorption spectra indicate a concentration decrease. Absolute values of RNA or protein per cell can at present accurately be determined only on isolated individual cells.

The following findings may be referred to: Increased *motor activity* (running until exhaustion) was found to cause a marked decrease in RNA and protein concentration in the motor root cells. It was therefore concluded that the functional activity of the nerve cell was accompanied by increased nucleotide and protein metabolism (Hydén, 1943a). Using X-ray microradiography, Gomirato (1952, 1954) found motor exhaustion to produce a marked decrease of the total dry mass, including the RNA-containing fraction per unit area in the motor root cells. Attardi (1953) found a significant decrease of the RNA content of the Purkinje cells after muscular exhaustion.

Moderate *sensory stimulation* by use of rotation caused an increase of the RNA concentration in the vestibular ganglion cells of around 40% and also an increase of the amino acid absorption (Hamberger and Hydén, 1949a). The same effect was found in the Deiters' cells; there also the results showed an increased nuclear concentration of nucleotides (Hamberger and Hydén, 1949b). The frequency distribution of the different types of nerve cells in the Deiters' nucleus showed a significant shift toward the groups with a higher RNA concentration. Prolonged, intense rotatory stimulation (8 minutes each day for 6 days) caused a decrease in the RNA concentration in the vestibular ganglion cells.

Taking the activities of cytochrome oxidase and succinoxidase as an index of the relative rates of intracellular oxidation, Hydén and Pigón (1960) made a study of Deiters' cells after moderate rotatory stimulation for 25 minutes each day for 7 days. The microdiver technique was used. Measured as microliters $O_2 \times 10^{-4}$ per nerve cell per hour, the relative activities were considerably higher in the stimulated cells. The ratio for cytochrome oxidase activity for stimulated cells : control cells was 1.6 ($6.7 : 4.2 \times 10^{-4}$), and for succinoxidase, 2.7 ($6.0 : 2.2 \times 10^{-4}$). When the dry weight was determined by the X-ray technique of control cells and stimulated cells, there was found a significant increase in weight of the stimulated cells, i.e., an increase of the proteins. This indicates that the effect of the stimulation was not only an increase of the enzyme activity but a real increase in enzyme concentration. With respect to the role of these enzymes in electron transport and coupled high-energy phosphate bonding, these findings point to an increased energy metabolism accompanying increased neuronal activity. They further show that the nerve cells adapt their respiratory rate to an increased functional level and activity.

The dry weight of the Purkinje cells in the flocculus was found also to increase considerably after rotatory stimulation (Hydén, 1952a). Acoustic stimulation caused a decrease in the nucleoprotein concentration in the spinal ganglion cells. The whole cycle of changes took 3 weeks under the conditions used (Hamberger and Hydén, 1945). The importance of the adequate stimulation for postnatal chemical development has been shown by Brattgård (1952), studying the retinal ganglion cells. When the animals received normal light stimulation the mass, i.e., the total amount of organic substance, increased 100% during the early postnatal period. This development did not occur if the stimulation was excluded.

Edström and Eichner (1958b) have studied the ganglion cells of the supraoptical nucleus after stimulation with sodium chloride. The amount of RNA in the cytoplasm and the nucleolus increased significantly with 80% and 35%, respectively. The RNA concentration of the nucleolus decreased significantly. There was a volume increase of the cell body, nucleus, and nucleolus. The findings indicated intensified RNA, and thus protein production, caused by the stimulation.

In a collection of nerve cells of a given type, e.g., Purkinje cells, anterior horn cells, Deiters' cells, spinal ganglion cells, two or more subgroups have been found. The nerve cells differ with respect to RNA, lipids, and proteins, or enzyme activity (Hydén, 1943a; Hamberger and Hydén, 1949b; Brattgård and Hydén, 1952; Gomirato, 1954; Edström, 1956a; Giacobini and Holmstedt, 1958). With regard to the importance of the RNA for protein production and the experimental results indicating protein production at increased neuronal function, the conclusion was that these differences depended upon the functional state (Hydén, 1943a, b, 1958).

The second probability is that quantitative or qualitative differences in the composition may reflect an existing biochemical specificity in the nerve cells (Hamberger and Hydén, 1953). Bodian (1950), Weiss (1950), and Gerard (1955) discuss and exemplify this question. Poliovirus attacks specifically motor neurons. In thiamin deficiency there is a degeneration of vestibular nuclei. Streptomycin attacks the same nuclei and causes changes in the RNA concentration (Floberg *et al.*, 1949). Various examples can be referred to. This also is a cytogenetic problem for further investigation.

A large body of information can be obtained from morphological studies on what Scholz (1957) terms "Stoffwechselmorphologie"—cellular changes following stimulation or due to other external factors. Some of them have been clarified by the above-cited studies. Among these may be mentioned especially the work of Dolley (1913), Einarson (1933, 1935, 1945), Krogh (1945, 1950), Einarson and Krogh (1955), and Einarson and Lorentzen (1946). Einarson developed a staining method with gallocyanine-chrome alum which he claims is a quantitative and stoichiometrical reaction for nucleic acids. He has found, as have other workers, that chromophobia of the nerve cells is the structural correlate of increased activity.

Summary. The experiments above referred to on the cytoscale and also, by inference, on the macro- and microscale, have demonstrated that RNA, proteins, and lipids are synthesized in the nerve cells at a rate that follows the neuronal activity. The metabolic rate of these substances seems to be high. It seems not farfetched to visualize the neuron as an enormous, glandlike cell, fulfilling its function under steady and rapidly changing production of proteins and lipoproteins, with the mediation of RNA as an activator and governing molecule. A tentative explanation as to why a production of conjugated proteins is linked with neuronal function is discussed on page 303, Section VI.

B. The Nerve Cell Mechanism for Protein Synthesis; the Nucleocytoplasmic Relationship

The chemical mechanism by which protein is formed in the cell is still a field for hypothesis (see page 304). Yet, a large amount of information has been obtained recently from studies on cell-free suspensions, virus, bacteria, protozoa, certain metazoan cells, e.g., thymocytes and liver cells. Studies on nerve cells are meager.

Cytochemical observations have indicated that RNA plays an important role in protein synthesis in cells (Caspersson, 1941; Brachet, 1942). This has now been shown also biochemically by blocking the RNA and by isotope studies. The present view encompasses the following points. DNA is necessary for protein synthesis in the nucleus, and even in the cytoplasm, seen on a long-term basis. It seems that the role of DNA in nuclear protein synthesis with respect to phosphorylation can be filled by

DNA from other tissues and also by RNA (Allfrey *et al.*, 1957). The nucleus has an energy-yielding system and one function of polynucleotides, whether DNA or RNA, may be to mediate the synthesis of ATP.

The activation of the nucleus seems to require a preliminary synthesis of RNA so that it incorporates amino acids into its proteins (Allfrey and Mirsky, 1957a, b).

There have been isolated DNA with a high molecular weight, approximately 40×10^6 and metabolically most active, and another DNA fraction with lower activity and weight (Levinthal, 1957). Likewise, there are two or more types of RNA in the nucleus. One fraction probably represents RNA of the nucleolus and is metabolically the most active; the other ribonucleoprotein complex is also very active (Allfrey and Mirsky, 1957b; Vincent, 1957). Using autoradiography, Ficq (1955) could show that the nucleolus especially has a high RNA metabolism.

Goldstein and Plaut (1955), using amoebas, indicated that labeled RNA probably passed from the nucleus into the cytoplasm. Plaut and Rustad (1957) found that this organism incorporated RNA in the cytoplasm also in the absence of the nucleus, indicating an independently working system. Besides the production of the nuclear protein, ultimately governed by a specific DNA and dependent upon the RNA, there is also a cytoplasmic protein production. This seems to be located to the so-called microsome fraction rich in RNA (Allfrey *et al.*, 1953; for a review see Brachet, 1957). As is pointed out on page 242, the small particles rich in RNA, the microsomes, may well be precipitation products, the RNA *in vivo* existing instead in more diffuse aggregates.

Be that as it may, the fraction of the cytoplasm rich in ribonucleoproteins is also the site of the protein production. The production of a specific protein requires a specific RNA. By analogy with the model of the DNA molecule, according to Watson and Crick (1953, 1954), the RNA should serve as a template for the protein. The sequence of the bases should determine the physicochemical and enzymatic properties of the protein formed, by determining the nature and position of the polar groups. An important fact is that the synthesis of proteins proceeds from free amino acids. They undergo condensation into polypeptide chains without the intermediate formation of complex peptides. The free amino acids are activated by reaction with ATP and enzymes (Hoagland *et al.*, 1956; DeMoss and Novelli, 1955). These activated amino acids are transferred to protein in an incorporation process which, according to Gale (1956), may be an exchange reaction between the activated amino acids and the amino acids already present in the protein associated with the RNA. Within a few minutes amino acids can be collected and assembled into a single molecule and released into the cytoplasm as a specific protein [see Loftfield (1957), who has also suggested an operating mechanism for this process]. The cited results and conclusions are based on results from studies on different types of material. A generalization may be hazardous, but the resulting picture is appealing.

The nerve cell offers unique possibilities to study changes of the nucleolus and nucleus and signs of chemical interaction between the nucleus and the cytoplasm. The following results have been obtained with quantitative cytochemical methods. They can be supported by many previous cytological findings on living and fixed cells. Detailed analyses have been made with UV microspectrography and X-ray microradiography of the large spinal ganglion cells of the angler fish *Lophius piscatorius* and other fish species (Hydén, 1943b, 1952b). These cells differ in size, and the largest contain considerably more RNA and proteins. The nucleolus of the large cells contain approximately three times as much RNA as do the smaller and contain relatively more proteins. It can be noted that Edström and Eichner (1958b) found a significant increase (80 %) of the absolute amounts of RNA in nucleoli of supraoptical nerve cells after stimulation compared with that in the control cell nucleoli. Hydén (unpublished) has observed that P^{32} is first incorporated in the nucleus of the nerve cell (Fig. 14, b).

In the nerve cells of *Lophius* containing more proteins, the measurements showed a gradient of 50 % in the nucleoprotein content from the nucleolus onto the folded part of the nuclear membrane. X-ray analyses showed this gradient to amount to around 30 %, computed on the mass near the nucleolar edge (see Fig. 15). On the outside of the nuclear membrane RNA and proteins were found in high concentrations. The nuclear membrane seemed to be unperforated, as seen through the masses of RNA. Since the small-type nerve cell seemed to be transformed successively to the larger type, characterized by an increased protein content, the production of RNA near the nuclear membrane was associated with the production of cytoplasmic proteins. The hypothetical conclusion was drawn that these nerve cells were characterized by a lively protein production. It was assumed that protein substances migrated from the nucleus to the folded part of the nuclear membrane, on the outside of which formation of nucleoproteins took place. It was also concluded that previous nerve cell changes interpreted as "Chromatin-Austritt" (Holmgren, 1899a), could be explained by this activity of the nucleolus.

An increase in the RNA concentration in the nucleus, especially in the chromocenter regions near the nucleolus, and the formation of RNA and proteins in high concentrations near the nuclear membrane, have been found after stimulation in other types of nerve cells in connec-

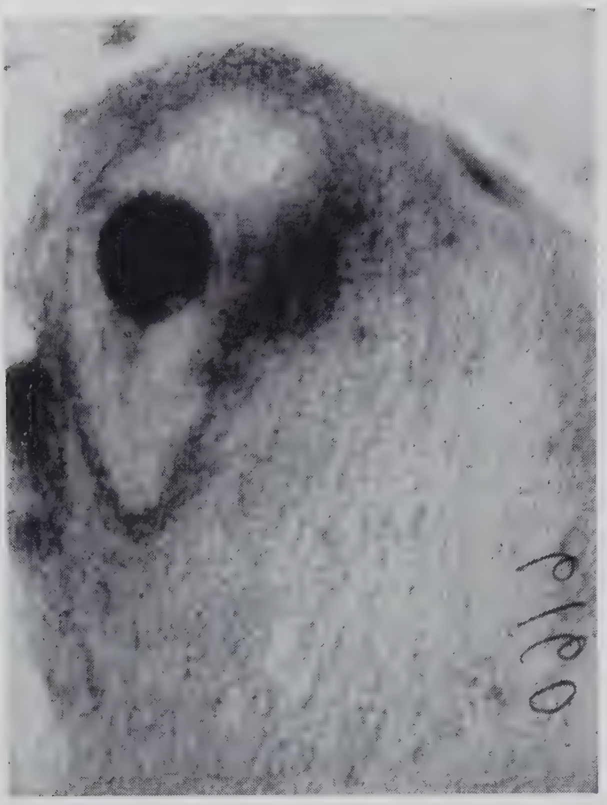


FIG. 15. Spinal ganglion cell of *Lophius piscatorius* photographed at 2570 A. From the nucleolus to the folded part of the nuclear membrane, a nucleoprotein gradient has been established. Magnification: ×1500. From Hydén (1943b).

tion with a concentration increase of RNA in the cell (Hydén, 1943a, 1947; Hamberger and Hydén, 1949a; Hochberg and Hydén, 1949). Confirmation that such findings indicate that the protein formation in the nerve cell is more active, comes from a study of nerve regeneration. The finding of nuclear membrane nucleoproteins is frequent in the stage when the amount of RNA has increased around 100% and the protein content has doubled (Brattgård *et al.*, 1957).

In nerve cell cultures, R. S. Geiger (1957) has observed particles detaching from the nucleolus and moving toward the nuclear membrane. They were seen to merge with the membrane. Lewis (1931) coined the term pinocytosis for the process, whereby cells engulf substances as droplets from the surrounding medium and transport them to another part of the cell. Holter and Marshall (1954) have studied pinocytosis in amebas. Palade (1956) discussed pinocytosis in endothelial cells, and Bennett (1957) considered this phenomenon to be a method of transport through cell membranes. It seems to the writer that the chemical observations in the nerve cells of *Lophius* may well be explained as signs of pinocytosis, whereby the nucleus exchanges larger complex of cellular substances with the cytoplasmic liponucleoproteins as well as other substrates. This interrelationship between nucleus and cytoplasm is no doubt mutual. Biochemical and biophysical, as well as cytological evidence, stress the nucleolus and attached regions in the nucleus as important centers for the intracellular nucleoprotein production.

An independent protein production in the nerve cell cytoplasm can of course not be excluded (Gersh and Bodian, 1943; Hydén, 1950) and is probably steadily going on. This can now be judged more safely on the basis of the results from the regeneration studies (Brattgård *et al.*, 1957). It may therefore be concluded that in the nerve cell body RNA, probably of both nuclear and cytoplasmic origin, is present.

A correlation between structural changes and functional activity in the nerve cells, indicating production of the Nissl substance by the nucleus, has been frequently noticed in important cytological studies (for review see Spielmeyer, 1922; Einarson, 1935, 1945; Hydén, 1943a, b; Vraa-Jensen, 1956; Scholz, 1957). Especially noteworthy are the studies of Holmgren (1899a), Scott (1899), Hatai (1901, 1902, 1904), and Einarson (1933, 1935), discussing migration of chromatin and nucleolar substances into the cytoplasm, and the production of Nissl substance through the activity of the nucleolus and the nucleus. In frog sympathetic ganglion cells, De Robertis (1954b) has described a rich accumulation of electron-dense particles within folds of the nuclear membrane and also the occurrence of holes in this double-lined membrane. The opinion of the present author is that such defects in a membrane at this high resolution might equally well be due to disruption caused by the cytological procedure.

The findings of a high nucleoprotein production in the nerve cells have been correlated with the results especially of Weiss and Hiscoe (1948) indicating a movement peripheral to the axoplasm. These authors conclude that there is a perpetual growth of the neuron with a steady replacement of catabolized proteins in the periphery. This production of new protoplasm should occur solely in the nerve cell body, and the finding of a high cellular protein production supports this view. For further discussion, see Weiss (1956) and Section IV on nerve regeneration.

C. The Nerve Impulse, Its Generation, Propagation, and Transmission

The capacity of the neuron to produce, propagate, and transmit impulses is one facet of the neuronal function. A proper stimulation gives rise to an excitatory or an inhibitory impulse, affecting other neurons through the synapses, or the effector organ through adjunction. The resulting bioelectric potentials, which are due to ion concentration gradients, are signs of activity. They give important insight into the multifold processes of the neuron, leaving out others equally important for the maintenance of the nerve function. One might ask if these potentials are more than secondary signs of activity, signals from the nerve cells which share the capacity to produce potentials with other excitable structures. The main speciality of the neurons lies in their capacity to select, store, and handle information for future recollection, and in this connection modulated frequencies may play an important role (see page 304).

The chemical changes in the neuron, so far discussed, that indicate metabolic processes take place within seconds, minutes, or hours. The action potential of the excited neuron rises and declines within milliseconds. The discussion of the generation of the nerve impulse and its transmission has centered on the ionic movements across the neuron membranes as the source of the electromotive force and the kinetics and energy sources involved. For the following discussion references are made to Hodgkin and Keynes (1955), Stämpfli (1954), Nachmansohn (1955), del Castillo and Katz (1955), Eccles (1957), Shanes (1957), to name a few.

1. The Saltatory Conduction

In a nerve fiber provided with a myelin sheath there occurs a discontinuous transmission of activity from node to node through local electrical circuits, the internodal parts serving for passive conduction. It has been shown that in peripheral nerve fibers the excitable membranes are confined to the Ranvier nodes. The highest thresholds for direct-current stimuli are found in the middle of an internode, the lowest at the node, where the axon often is constricted to 50% of the internode diameter. The unique structure of the nodes may also be recalled, the axon surface from myelin lamellae only loosely covered by the thin processes of the Schwann cells. When activity occurs at the nodes, there is a short initial outward current followed by a reversal of the membrane current. This type of conduction is most economical. It reduces the amount of sodium necessary to conduct an impulse to 1/300 of that exchanged in a nonmedullated fiber of the same diameter and reduces the heat production per impulse still more. Saltatory conduction seems also to assist in synchronization of impulses conducted in adjacent fibers.

2. Ionic Movements and Studies with Microelectrodes

The membrane theory postulates that the neuron is surrounded by polarized membranes and charges positively on the outside, negatively on the inside. The outside concentration of Na^+ ions is 150 m*M* and the inside about 15 m*M*. Corresponding values for K^+ ions are 5.5 m*M* and 150 m*M*, and for Cl^- ions, 125 m*M* for the outside concentration and 9 m*M*, for the inside. This causes a resting potential of –70 m. for large motor neurons. The specific membrane capacitance is 17.5 μF./cm.^{2}. The membrane resistance is 0.8 megohm. With a neuronal surface of 5×10^{-4} cm.^{2}, this gives a specific membrane resistance of 400 ohms per square centimeter. The membrane capacitance suffers no change during an impulse. Therefore, it seems that changes in the membrane potential are attributable to ionic movements across the surface membrane.

The ions move freely by diffusion or they are driven by electrochemical gradients (see Teorell, 1953). In the resting state, energy is needed to preserve these concentration gradients of K^+ and Na^+ ions. The electrochemical potential for Na^+ is 150 mv. and in the opposite direction to K^+ ions, for which has been found –20 mv. At the resting potential of –70 mv. the situation can be maintained only if the deficiency in inward flux is counteracted by an inward pumping mechanism. There seems to be an inward pumping of K^+ ions coupled with the outward pumping of Na^+ ions.

When the neuron is excited, there is a sudden release of electric energy, the axon potential, which occurs when the resting potential has been decreased to about –45 mv. The ionic permeability increases and Na^+ ions flow into the fiber, rapidly reducing the resting potential. This leads to a depolarization, or actually a reversal, of the membrane potential. The inward concentration gradient of Na^+ ions will eventually be balanced, according to Ling (1952), by fixed anion charges on the proteins inside the axon. According to this hypothesis, the movements of an ion occurs from fixed charge to fixed charge, as in an ion exchange resin. There will be a conversion of a high Na^+ conductance into a high K^+ conductance, the movements occurring with the concentration gradients and the Na^+ ions preceding in time. Then there is a rapidly rising action potential of 110 mv. of 1.5 millisecond's duration for large *motor* neurons, associated with the rapid inrush of Na^+ ions. The following phase is slower and is explained by the decrease in K^+ concentration when K^+ moves out. The restoration of the intracellular ion concentration involves extrusion of Na^+ and the uptake of K^+ by the pumping mechanism. This occurs against the concentration gradient and requires energy. Only the restitution of K^+ ions seems to be associated with energy consumption. Using an isotype technique, Keynes and Lewis (1956) could support the sodium hypothesis by showing that the movements of Na^+ and K^+ are large enough to alter the potential across the nerve membrane. The Na^+ pump can further be interrupted by, for example, dinitrophenol. By injecting ATP into cells where the ATP synthesis was inhibited, Caldwell and Keynes (1957) found that the rate of Na^+ extrusion could be restored to a certain degree, but only temporarily. After the impulse the fiber is unexcitable for 0.5–1 millisecond, the absolute refractory time. Abood and Abul-Haj (1956) suggest that a complex of protein and hyaluronic acid is involved in ionic accumulation and that the ionic transport during activity is due to structural alteration within the complex.

In this way successive parts of the membrane are activated, the small potential change of 15 mv. becomes amplified, and the impulse is propagated along the axon without loss of signal strength but stops at the presynaptic membrane. The function of the myelin sheath seems to be to cause the impulse to skip from node to node in a saltatory manner. An important fact is the amplification of the potential change. If energy is needed for the pumping mechanism, probable sources are the ATP and oxidative processes in the mitochondria (Hodgkin and Katz, 1949a, b; Hodgkin, 1951; Hodgkin and Huxley, 1952). Lüttgau (1956b), among others, assumed a carrier system responsible for the transport capacity in a resting fiber and is activated at depolarization. Von Muralt (1957a) and Kuntz (1956) considered thiamin and its rapid oxioreduction as a governing factor for the movements of Na^+ ions.

The nerve cell is densely covered by synaptic endings. The sum of many impulses easily exceeds the threshold of excitation. Where is the postsynaptic potential generated, which is the trigger mechanism, and which is the mechanism for the transmission through the narrow cleft of 150–200 A. between the pre- and postsynaptic structures? The microelectrode technique and nature's offer of an ideal experimental set-up in the form of the giant fibers have given the field a good start. A single presynaptic stimulation generates an excitatory postsynaptic potential. By summation of many elementary excitatory potentials, the threshold for the neuron may be exceeded and an impulse discharged. The impulse starts in the first, unmyelinated part of the axon at a threshold of 6–18 mv., generating a small potential. In the rising phase of the potential there is an inflection attributable to this impulse. The large potential, according to Eccles (1958), is supposed to be generated indirectly when the impulse invades the soma-dendritic membrane. It takes 20–40 mv. to get a depolarization sufficient to start an impulse from the soma or the dendrites. The current hypothesis therefore says that synaptic excitatory action generates an impulse solely by the depolarization of the neuron, i.e., by producing an excitatory postsynaptic potential. The depolarizing current flows inwards, across the subsynaptic membrane, so that a return current may flow outward, depolarizing the remainder of the postsynaptic membrane. The dendrites have electrical properties similar to those of the soma and conduct the same type of impulses (Eyzaguirre and Kuffler, 1956).

According to a hypothesis of Eccles, there are only excitatory cells, forming the peripheral afferent and efferent pathways, and inhibitory cells of Golgi type II. An inhibitory impulse should give an electric mirror image of the excitatory potential, depressing this below the threshold level for the generation of an impulse and creating a hyperpolarization.

On the problem of transmission there are two schools of thought. The different views are phrased in rather uncompromising sentences. It seems to an observer, however, that these views could merge, with just a little increase in knowledge. Dale (1933) found that acetylcholine (ACh) is released from motor nerve endings and can produce muscle impulses. A single preganglionic volley can liberate around 10^{-15} g. ACh around one nerve cell (Eccles, 1953). ACh is rapidly hydrolyzed by a specific cholinesterase. This explains the 1–10-millisecond duration of the depolarizing current (Eccles, 1957). Acetylcholinesterase is localized in the sheath of the fiber, but absent from the axon (Boell and Nachmansohn, 1940). That it is necessary for conduction has been shown by the use of specific inhibitors.

According to Nachmansohn, the propagating agent along the nerve fiber, as well as across synaptic junctions, is electric current. The action of ACh occurs within the conducting membrane and is an intracellular process. ACh exists in a resting condition in a bound and inactive form. When the membrane is excited ACh is released. The free ester acts upon a specific receptor, a protein, produces the change in ionic permeability, and generates the potential. The free ester is then attacked by acetylcholinesterase. The receptor returns to its resting condition and the barrier to the ion movements will be re-established. In study the effect of tertiary and quarternary nitrogen compounds, Altamirano *et al.* (1955) concluded that the presence of a methylated quarternary nitrogen group is decisive for the effective combination of ACh and the receptor. The energy for the restoration process comes from the metabolic glucose cycle. The fact that ACh is found at the synapses is, according to Nachmansohn, due to lack of a barrier there against ACh as a methylated quarternary ammonium salt. ACh is produced in the whole neuron, transported peripheral to the synapse, and leaks out only there. Considering the high protein-producing capacity of the neuron, like an enormous glandular cell, to the present author this view of ACh production is attractive. As one product among many, ACh may be locally active where it is released, whether centrally or in more peripheral parts.

The other view on the transmission problem has many supporters (e.g., Hodgkin, Huxley, del Castillo, Katz) and maintains, with much evidence, that the transmission is mediated by a transmitter substance—ACh. Grundfest (1957) found that all data strongly indicate that postsynaptic activity is not induced by electrical stimuli. ACh released at the presynaptic membrane reaches the postsynaptic membrane by diffusion, reacts with specific receptors on this surface, and causes a depolarization. Thus, within the boundaries of a neuron, the impulses are spread by electric currents. At the synapse these are broken and the local circuit action of the axon is replaced by short-range diffusion of ACh. To account for the free and bound form of ACh and the phenomenon of spontaneously occurring subthreshold miniature potentials, storage of ACh is supposed at the synapses, as well as a release mechanism (del Castillo and Katz, 1955; Birks and McIntosh, 1957; Eccles, 1957). Such miniature potentials occur at the end plates of resting muscles and are supposed to be due to the release of 100 to 1000 molecules of ACh. The normal end-plate potential is set up by release of a great number of such "quanta" of ACh. The ACh is supposed to be bound to the 100–400 A. vesicles of the presynaptic axoplasm. These could move close to the presynaptic membrane. A propagated disturbance would then cause the vesicle to merge with the membrane and the ACh to be ejected into the synaptic interspace. Bennett (1956) visualizes this process as part of generally occurring membrane vesiculations, serving as ion pump mechanisms. It might be noted that evidence has been produced that vesicles may be formed by pinching off from membranes also in, among others, endothelial cells and macrophages. Also, Eccles and Fatt (1956) pointed out that intracellular recording so far has failed to reveal miniature synaptic potentials of ganglion cells. Therefore, however appealing the current idea of the synapse as a device for applying minute amounts of a transmitter substance on the postsynaptic membrane might be, the mechanism is still an open question (see also discussion of the vesicles on page 260).

ACh is synthesized by choline acetylase, which transfers acetyl groups from coenzyme A to choline. ATP provides energy for the acetylation of coenzyme A, the whole process being thus intimately associated with the mitochondria. Weiss (1944) supposed that choline acetylase is manufactured in the nerve cell body and carried toward the periphery by axoplasm movements.

The central nerve tissue consists not only of the neurons but also of the neuroglia. As is stated below, the number of glia cells surpasses that of nerve cells manyfold. Whether the total volume of the glia amounts to that of the neurons or is even larger, is still an open question but remains a possibility. With respect to the morphological and functional relationship between glia and neurons, it is therefore interesting that glia is capable of electrical responses to direct stimuli. Slow, reversible potential variations have been recorded by the microelectrode technique from glia both *in vivo* and in glia cultures (Tasaki and Chang, 1958).

The response consisted of a sudden depolarization followed by a slow return to the resting level. The potentials from astroglia were of about a thousandfold longer duration than those recorded from nerve cells. In this connection it was pointed out that Chang and Hild (1959) found electrical stimulation to evoke a slow mechanical contraction in glia. It is not surprising per se that the glia could produce bioelectrical potentials. But it may prove to be of significance for the neuronal function, placed as one bit of information into the mosaic which has begun to be formed of the function of the glia in relation to the neurons.

D. The Interaction between Nerve Cells and Glia Cells

The glia cells outnumber the nerve cells by a factor of around 10. There are many morphological and a few chemical data that indicate a functional relationship between glia and nerve cells.

In the discussion of the neuropile and the "extracellular fluid space," page 221, it was stressed that the space of the neuropil seems to be completely filled with the delicately folded processes of the astrocytes and oligodendrocytes and those of the dendrites. The only "free space" available seems to be the 100-A. cleft visible in electron micrographs. That these clefts in the picture really represent "free spaces" filled only with water and solutes, remains to be proved.

To the glia cells, astrocytes, and oligodendrocytes, possibly in association with the endothelial cells, has been attributed the so-called blood-brain function; for a discussion of this problem the reader is referred to the Symposium on "The Biology of Neuroglia" (1958). Studies at high resolution have not revealed any morphological differences between endothelial cells from brain capillaries and those from other organs (Maynard *et al.*, 1957; Bennett, 1957). The end-feet of the astrocytes insheathe the brain capillaries to about 85%. The remaining part is covered by processes from oligodendrocytes and neurons. The "blood-brain barrier effect" found earlier can better be explained as the result of the cellular permeability in conjunction with the relatively small capillary area which exists in the brain. Brattgård and Lindqvist (1954), using Br^{82}, showed, e.g., that the difference in activity between cortex and white matter could satisfactorily be explained as being due to the difference in vascularization. In spite of the poor vascularization, gas exchange in the brain is assured by the large amount of blood passing the brain per unit time. The selectivity of the barrier for different ions and molecules remains, however, to be explained. That the glia cells may be active in this respect cannot be excluded.

Wilke and Kircher (1952) and Bairati and Bartoli (1955) have studied the birefringent glia protein components. Bairati identified the glia protein as an α-keratin.

The following discussion centers on the view advanced by the author and associates (Hydén *et al.*, 1958; Hydén, 1959; Hydén and Pigón, 1960) and based on the relative activity of respiratory enzymes in nerve cells and glia cells, that glia cells may be energy donors to the nerve cells. It is reasonable to suppose them to function as such by pinocytosis, the mechanism by which cells engulf small droplets from the surrounding medium.

Glia cells in cultures. Both astrocytes and oligodendrocytes show rhythmic pulsatile activity (Pomerat, 1951, 1955; Lumsden and Pomerat, 1951). This was thought to produce a massaging effect on the nerve cells and, with respect to oligodendrocytes to influence the nutritional state of the axoplasm. *In vivo* such activities might assist the propulsion of interstitial fluid. Particularly important seems to be the observation that the processes of astrocytes form interlacing feltworks and show delicately undulating membranes. The tips of these membranes showed uptake of fluid by pinocytosis (Pomerat *et al.*, 1957).

The mass of glia cells. The bodies of the glia cells are hard to stain. At high resolution they appear empty, especially the astrocytes. This can no doubt be explained by the destructive effect of cytological procedures, fixation, embedding, and staining, which remove, it seems, a large part of the organic substances in these cells. Determined per volume unit, the weight of fresh, frozen, and dried hypoglossal and Deiters' cells is around 0.20 pg./μ^3. The mass of the glia cells turned out to give the same value, 0.20 pg./μ^3 (Hydén, 1958, 1959).

Extraction with pure chloroform gave a larger loss for the glia cells than for the nerve cell bodies, indicating that glia cells contain more free lipids.

Morphological relationships. From careful cytological observations, Holmgren (1901, 1904a, b, 1914) concluded that glia cells not only encapsulate the nerve cell, but also establish an intracellular contact with the nerve cell body. He interpreted his picture to show that osmophilic granules were supplied to the nerve cell from the glia cells, and concluded that the glia cells had a nutritive function, were trophocytes. The connection between neuroglia cells and capillaries through end-feet is a well-known fact.

Scheibel and Scheibel (1955, 1958), in sections stained with the Golgi technique, described the occurrence of contacts between oligodendrocytes and nerve cell bodies, dendrites and terminal portions of axons, on the one hand, and with astrocytes on the other hand. The same axon could then innervate both the postsynaptic neuron and its oligodendrocytes. The processes of the astrocytes may also terminate on oligodendrocytes. Noteworthy is the well-known capacity of glia cells to react to damage to the nerve cells, e.g., during retrograde reaction (Spielmeyer, 1922; Cammermeyer, 1955).

Submicroscopical structure. This problem has lately been dealt with by Palay (p. 24) and Dempsey and Luse (p. 99) in "The Biology of Neuroglia" (1958), by Wyckoff and Young (1954), Luse (1956a, b), Farquhar and Hartmann (1957), Schultz *et al.* (1957), and Niessing and Vogell (1957). The astrocyte has a uniformly granular nucleus and nucleoli may be present. Luse (1956b) and Dempsey and Luse (1958) described the astrocyte cytoplasm as scanty but elaborated in complexly folded processes containing meager amounts of cytoplasmic structures. Schultz *et al.* (1957) emphasize the "empty" impression made by atrocytes, with only traces of content left after the fixation. The oligodendrocytes resemble rather the nerve cells, according to Schultz *et al.* The glia cells have fewer mitochondria than have nerve cells.

All interstices in the neuropil are filled with the multifolded processes of the astrocytes and by the oligodendrocytes. The nerve cell bodies, dendrites, neurites, and oligodendrocytes seem to be surrounded with the delicate processes of the astrocytes. They frequently establish contact with the neurons on the one hand and with capillary walls on the other and are closely applied to the capillary basement membrane. The capillaries are insheathed by glial processes. Thus the nerve cell is clearly isolated from the immediate blood supply by the glia. As was emphasized earlier, the only extracellular space seems to be the 100–200 A.-wide "spaces" which separate the protoplasmic membranes. Wyckoff and Young (1954, 1956), Farquhar and Hartmann (1957), and Schultz *et al.* (1957), like Holmgren, point out that the morphological relations suggest that the astrocytes may serve as the principal transport system to the nerve cells.

The findings of Hess (1958) show a particularly close relationship between neuroglia cells and their nerve cell, and bear out the general morphological conclusion by Holmgren. Hess found that the processes of the glia cells form a thin sheath around the nerve cells consisting of several overlapping layers. The inner layer of the sheath penetrates into the neuron as invaginations to which the appearance of the trophospongium of Holmgren would seem to be attributable. The glial cytoplasm is, however, always separated from the neuronal cytoplasm by two "membranes."

Enzyme activity. Using the microdiver technique, Hydén *et al.* (1958) and Hydén and Pigón (1960) studied the activity of cytochrome oxidase and succinoxidase in spinal ganglion cells and their capsules containing the glial satellites as an indication of the respiratory rate. Determined per unit volume, the relative activity of cytochrome oxidase was 0.9 for the nerve cells as compared with 1.6 for the glia cells. Corresponding figures for succinoxidase were 0.7 for the nerve cells and 5.1 for the glia cells. Thus both enzymes exhibit higher activities per unit volume of glia cells. The glia cell : nerve cell ratio for succinoxidase activity was 7, as compared with approximately 2 for cytochrome oxidase.

Deiters' nerve cells and their perineuronal and adjacent glia cells were also determined with respect to these enzyme activities. The glia is mainly of the oligodendro-type with an admixture of approximately 10% astrocytes. The ratio for cytochrome oxidase (nerve cell : glia cells), determined for the same volume, was 2.7 ($11.5/4.2 \times 10^{-4}$ μl./O_2/hr. per unit volume). For the succinoxidase activity, the ratio was 2.0 ($4.5/2.2 \times 10^{-4}$ μl./O_2/hr. per volume unit).

If the findings of Lowry *et al.* (1956a) on the enzyme activity in spinal ganglion cells and their capsules containing glia cells are computed on the same basis, assuming that the volume of the spinal ganglion cell is approximately five times greater than that of the glia cells, the following is found: The activity of phosphoglucoisomerase is five times higher in the glia cells per unit volume; that of glutamic-aspartic transaminase, five times, and that of glutamic dehydrogenase, four times higher than these activities in the nerve cells.

In order to study the reaction of the (oligodendro) glia at increased functional demands, the enzyme activity was measured in vestibular nerve cells and in the same volume of glia cells after vestibular stimulation (Hydén, 1959) (see details page 275). The ratio glia cells : nerve cell for cytochrome oxidase changed from 2.7 for the controls to 0.4 for the stimulated animals. The corresponding values for succinoxidase were 2.2 and 0.65, respectively. With respect to the enzyme activity of the glia cells from the controls, compared with that from the stimulated animals, a significant decrease was found in the cytochrome oxidase activity. Summarizing, the stimulation caused an increase of the respiratory enzyme activity of the nerve cells. At the same time the enzyme activity of the glia cells decreased, and significantly so for the cytochrome oxidase. These results are at present impossible to interpret. A tentative working hypothesis is, however, presented at the end of this section.

Thus, these experiments may indicate a higher respiratory rate and amino acid metabolism in the oligodendroglia cells per volume unit, compared with that of the nerve cells. They may further indicate a certain specialization, i.e., a division of labor between the nerve cells and their satellites. Either the glia cells consume a large amount of energy, or they may be concerned with supply of energy to the nerve cells. It seems important that such comparative studies be performed on nerve cells and isolated glia cells which actually surround these nerve cells. Marginal glia cells or these from cauda equina may well have another functional purpose.

In previous sections are mentioned morphological observations showing that the nerve cells are intimately surrounded by, and associated with, glia cells and their processes. More indications of the glia cells as a transport or "mediating" system between the capillaries and the nerve cells also exist. De Robertis and Bennett (1954a) described small vesicles 350–650 A. in diameter in the endothelial cells of the capillaries and in oligodendrocytes of ganglion cells. The vesicles were interpreted as being fluid globules. Referring to the original work of Lewis (1931) and to the assumption of Palade (1953) that these vesicles transported fluids across the capillary wall by pinocytosis, they also assumed that the glia cells mediated exchange between the nerve cell and the intercellular space by pumping material in vesicles from one surface of the cell to the other. In this connection the old observations of Holmgren are relevant. Bennett (1956) has made further observations on a possible way in which such vesicles may be formed. In the ameba, there can be seen a wrinkling of the plasmolemma, followed by an invagination resulting in tunnel-like formation into the cytoplasm at pinocytosis (Brandt, 1958). Bennett (1956) drew the conclusion that the vacuole membrane is derived from the plasmolemma and that proteins are adsorbed to this membrane.

When data obtained with macrochemical methods on glia tumors or with quantitative histochemical methods on cortex are considered, the results do not entirely agree with those from studies on the cytoscale. Only with regard to the oligodendroglia does a conformity exist. It seems relevant to point out that the resolving power of the first-mentioned methods does not permit a correlation between enzyme activity and structures; this is done by inference. In the careful studies by Pope *et al.* (1956), it is pointed out that the activity of respiratory enzymes in layer VI in the cortex is less than in layers I–V, i.e., decreasing when the layers become more infiltrated with fibers and glia cells and the population of nerve cells and dendrites is less. They conclude that the high oxidative metabolism of the cortex is not contributed to importantly by any of the neuroglia cells. Pope (1958) summarizes evidence for this view as follows: There is a higher O_2 consumption in gray matter compared with white. A smaller requirement of the glia cells for O_2 is indicated by a relatively smaller capillary bed per unit volume in the white matter. Glia is relatively resistant to hypoxia. There are fewer mitochondria in glia cells than in nerve cells. Victor and Wolf (1937) and Heller and Elliott (1955) have found a low rate of O_2 utilization in gliomas, the oligodendrogliomas having a higher oxidative metabolism than astrocytomas. On the other hand, Abood *et al.* (1952) found in glia cultures high O_2Q values and approximately the same cytochrome oxidase activity in glia cells and in nerve cells. Foster (1956) found that the Schwann cells account for approximately 25% of the respiration of the squid fiber. The results of Pope indicate that proteolytic activity in the glia cells are of the same order as that of neurons. Pseudocholinesterase has been located in glia by Koelle (1954). Carbonic anhydrase has been found in glia by Tschirgi (1952), who assumed that it is concerned with electrolyte exchange between serum and cerebral fluids. Klüver (1944a, b, 1955) has found coproporphyrins in white matter of the CNS and assumed a localization in the glia cells. Robins *et al.* (1956) concluded that purine nucleoside phosphorylase, aldolase, and lactic dehydrogenase are localized in the oligodendroglia. They estimated that on the whole the neurons are five times as active as an equal weight of glia.

Summary. A working hypothesis on the function of glia cells on the basis of current observations could be phrased in simple words, corresponding to the poverty in knowledge. The glia cells may be functional appendages to the neurons. They mediate the transport of substances from the capillaries to the nerve cells by pinocytosis and thus take part in the blood-brain barrier mechanism. The glia cells prepare substrates for the nerve cells, metabolizing them to a certain degree so that only the last steps, including the conversion to energy, remain for the nerve cells, and thus function as energy donors to the neurons. Why, then, is the enzyme activity inhibited at increased neuronal activity? An explanation may be that at periods of heavy functional demands, when the nerve cells demand more energy, they are given priority to the electron transporting system by the neuroglia. This is reflected in the decreased cytochrome oxidase activity reported above. There may be an inhibiting mechanism at that terminal step at increased neuronal activity. The glia may be the cellular parts of the CNS which then have to resort to substances other than glucose. The clinical implication, if such a function of the glia cell could be proved, is clear. Mental disease could as well be thought to depend upon a disturbance of processes in glia cells as in the nerve cells.

IV. Regeneration of Peripheral Neurons

A. *General Features*

Regeneration is an inherent characteristic of peripheral neurons and comprises the morphological and functional reconstitution which can be repeated over and over again during a life cycle. There is a vast literature on the morphological changes (Ramon y Cajal, 1928; Nageotte, 1922; Boeke, 1935; Young, 1942, 1950; Weiss, 1950; Guth, 1956) and an analysis of important factors in the outgrowth mechanism (see Weiss, 1956). In several respects the regeneration is a repetition of the embryonal outgrowth of the neurite, and data from this field are most important (Hamburger, 1950, 1955). There are only a few observations of pertinent central chemical changes (Hydén, 1943a, 1947, 1955a, Brattgård *et al.*, 1957; Gersh and Bodian, 1943; Bodian, 1947). The discussion that follows will be centered on data which can be used for a temporary hypothesis of a cytophysiological concept of this regeneration; Wallerian degeneration will not be discussed.

Nissl (1892) described the morphological changes which began a day after the severance of the nerve in the facial nucleus as "primäre Reizung," later they were called retrograde (Marinesco, 1897; van Gehuchten, 1897; Lugaro, 1895). They consisted in an increase of the cell surface, with a swelling of its contours, a displacement of the nucleus, and a dissolution of the Nissl flakes into small, basophile dustlike particles, a chromatolysis. These changes were very marked after a week, but disappeared successively. Nissl substance was reproduced also in the form of basophile masses near the nuclear membrane, and the original cell picture was reconstituted after 50–60 days. From the stage of maximal changes about 2 weeks after the damage, the nerve cell can be destroyed, develop into a constant atrophic state, or be repaired. The chromatolytic changes are inversely proportional to the distance of the trauma to the cell body. In the periphery, the axon will undergo degenerative changes which also develop in a short part of the proximal stump. Already 3 hours after the trauma, thin sproutings appear at the cut end of the nerve, but these are abortive. During the second day new axons begin to traverse the scar tissue for approximately three more days. Thereafter they grow out, richly ramifying and with a velocity of 3–4 mm. per day for most mammal myelinated nerves. The Schwann cells at the central end of the fiber and throughout the peripheral part begin to proliferate and to grow in size. They are believed to be the main guiding structures for the outgrowing axons. The outgrowth of axons has been studied with time-lapse cinematography (Lewis, 1950; Hughes, 1953; Peterson and Murray, 1956; Nakai, 1956). Already in 1910, Harrison described the axon growth as a type of ameboid movement. The axon seems to be a thin tube comprising an outer gel layer and an inner, less viscous core. The axon tips advances, constantly changing its pattern of terminal branches with thin, undulating membranes. Uptake of fluid through these by pinocytosis has been described (Hughes, 1953; Nakai, 1956). The outgrowth was considered to occur by the conjoint action of the uptake of fluid and an addition of axoplasm by the distal flow. Nakai observed vacuoles moving with a velocity of 1–3 μ per minute toward the perikaryon.

One main branch carries the growth farther and others are obliterated. Weiss considers the growth to occur by peripheral transportation of new material in the axon, which shows peristaltic movements and adds the tip. He has discussed different factors of importance in the outgrowth process. Oriented structures, e.g., in connective tissue, were believed to guide the axons. By applying a constriction around a regenerating nerve, Weiss and Hiscoe (1948) found deformations proximal to the constriction in the form of localized dilatations. These configurations were interpreted as damming up the axoplasm owing to an increased resistance to the movement of the axoplasm. When the constriction was released, the dilatations disappeared and the narrow distal part of the fiber widened. The rate of descending axoplasm after release was found to be 1 mm. per day. The observed nucleoprotein gradient from the nucleus outward in the cytoplasm and indications of high protein production in regenerating nerve cells (Hydén, 1943a) have been taken to support the view that axoplasmic proteins are manufactured in the nerve cell soma and transported peripherally. Samuels *et al.* (1951) found indication in regenerating nerves of a peripheral flow at a rate of about 2.5 mm. per day of a phosphoprotein; the compound was thought to be inositol metadiphosphate. The concentration of phosphoprotein decreased (Logan, 1952). Weiss considered nerve regeneration to be a special manifestation of a peripheral growth process steadily going on in the neurons (Weiss, 1944) and expressing itself in the centrifugal movement of axoplasm. Other structural facts have also been taken as reflecting the effect of such a movement. On either side of the nodes is a local swelling of the fiber; the proximal bulb is greater in diameter than the distal one (Lubińska, 1954). The mean diameter of the initial half of the internode has been found to be slightly smaller than that of the terminal half (Lubińska and Lukaszewska, 1956).

Ramon y Cajal (1928) assumed that the outgrowing fibers are directed into the peripheral fiber portion by chemical attraction. Weiss stressed the importance of oriented structures in the tissue on the basis of the experiments and dismissed the idea of chemotropism. There are some observations, however, that leaves this still an open question. Sunderland (1953) found that, in opossum, regenerating fibers could bridge a gap of 35 mm. and find a complicated way to the distal stump (in the ulnar nerve). Levi-Montalcini and Hamburger (1953), and Levi-Montalcini *et al.* (1954) found a specific agent, possibly pentosenucleoproteins, in mouse sarcomas which selectively stimulated outgrowth of spinal and sympathetic ganglion fibers. Finally, collateral nerve regeneration (Liu and Chambers, 1956) is assumed to depend partly on chemical agents released from the degenerating end plates. Von Muralt (1946) has found a thiamin-like compound to be liberated at the distal end of a cut nerve. Lubińska (1956a) studied mechanically important factors at the outflow of axoplasm.

Peterson and Murray (1956) studied myelin formation in cell cultures and concluded that both the nerve cell and the Schwann cell are necessary to the elaboration of the myelin sheath. The appearance of regenerating myelin was similar to that of embryonic nerve (Terry and Harkin, 1957).

There are various factors that control the growth of the whole neuron. When the neuron is disconnected from its effector organ it decreases in size and increases again with reconstituted connection. A larger peripheral field to innervate increases the size of the neurons. Especially Hamburger (1950, 1955, 1956) has studied the adjustment of the neurons during development to their functional demands through proliferation, differentiation, and elimination. He shows that the neurons are very adaptable to functional demands and easily change their size, as is also stressed on page 222.

If the thin axons reach the peripheral organ they will continue to grow in diameter during the maturation period, and the spectrum of axon diameters characteristic for the nerve will be restored (see Young, 1950). After a delay of less than 10 days after the arrival of the fibers, the functional connection begins to work. The maturation period takes a long time—morphologically around 200 days in motor nerve fibers (Young, 1942; Gutmann and Sanders, 1943). Electrically normal conditions were not restored even after 180 days. The fiber diameter was small and the conduction rate lower than normal (Collins *et al.*, 1955).

B. *Cytochemical Observations*

Chemical determinations in amount per cell have shown that great production of RNA, proteins, and lipids occurs during regeneration (Brattgård *et al.*, 1957). The changes are different during the outgrowth and the maturation period and can be correlated with morphological changes. During regeneration the nerve cell replaces fifty to a hundred times the organic material contained in the cell body. That means for a large nerve cell approximately 2 μg. and for a smaller cell about 0.1 μg. Since data indicate also increased protein and RNA metabolism in the nerve cell during regeneration (Fischer *et al.*, 1958; Brattgård and Hydén, 1960), the total production of nucleoproteins is certainly large.

The latent and the outgrowth period. During this stage the thin axons slowly traverse the scar tissue and rapidly grow out to the effector organs, e.g., in the case of a motor nerve. The chromatolytic reaction rapidly develops. Already after the first day, the concentration of nucleoproteins

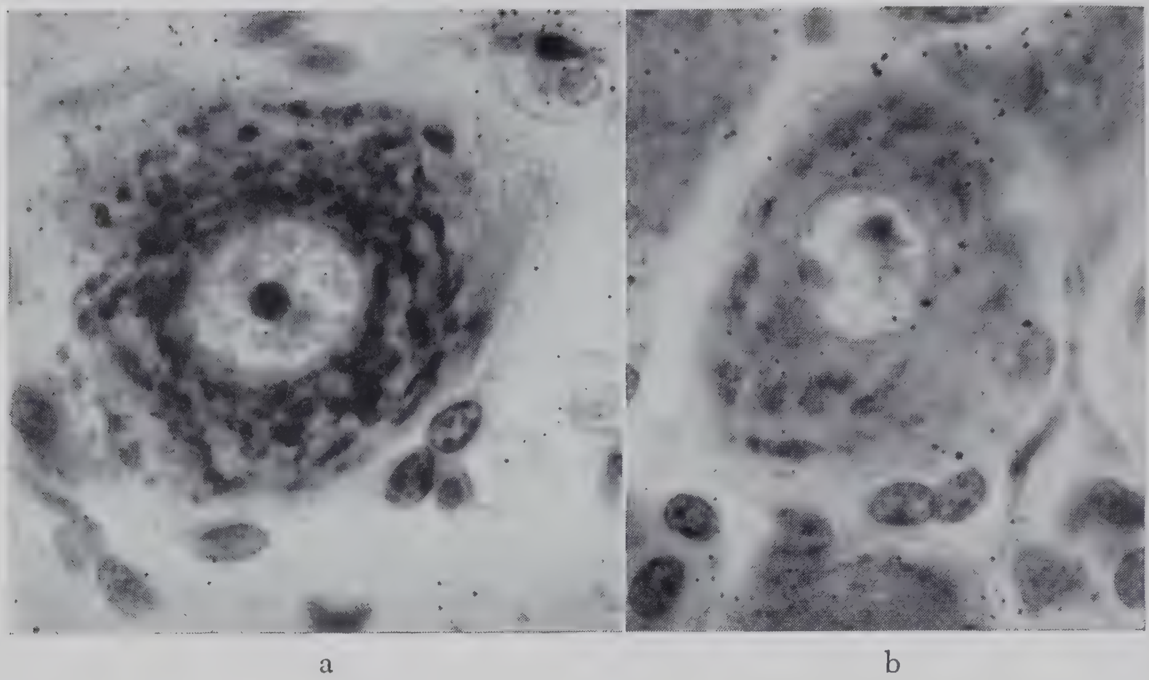


FIG. 16. a. Control nerve cell from ganglion nodosum. Photographed at 2570 A. Magnification: × 1370. b. Nerve cell from ganglion nodosum 19 hours after sectioning of the axon. Note the decreased absorption at 2570 A. within the cytoplasm, especially within the lower sector at right. Magnification: × 1350. From Hydén (1943a).

decreases within a sector of the cytoplasm (Fig. 16). After a week, when the thin $\backsim 0.5\,\mu$-thick axons grow at a rate of around 3 mm. per day, the RNA concentration is very low in the cytoplasm. The UV microspectrographic studies cited are those of Hydén (1943a, 1955a), Gersh and Bodian (1943), and Bodian (1947). The quantitative results quoted are taken from Brattgård *et al.* (1957). In some nerve cells RNA appears as a crescent around the part of the nucleus facing the largest cytoplasmic mass.

The central part of the nerve cell, weakly absorbing at 2600 A., was characterized by a strong positive reaction for acid phosphatase (Bodian and Mellors, 1947; Bejdl, 1954). Howe and Mellors (1945) found decreased cytochrome oxidase activity in such cells. The nucleolar satellite moves slowly away from the nucleolus toward the nuclear membrane during the retrograde changes. It was found later to return. The nucleolus was found to enlarge during the regeneration (Crouch and Barr, 1954). Outgrowing nerve fibers showed a strong cholinesterase reaction according to Koelle's method (Snell, 1957). The protein-bound phosphorus and other phosphorus-containing fractions in regenerating nerves were increased and showed increased specific activity (Logan, 1952; Bodian and Dziewiatkowski, 1950).

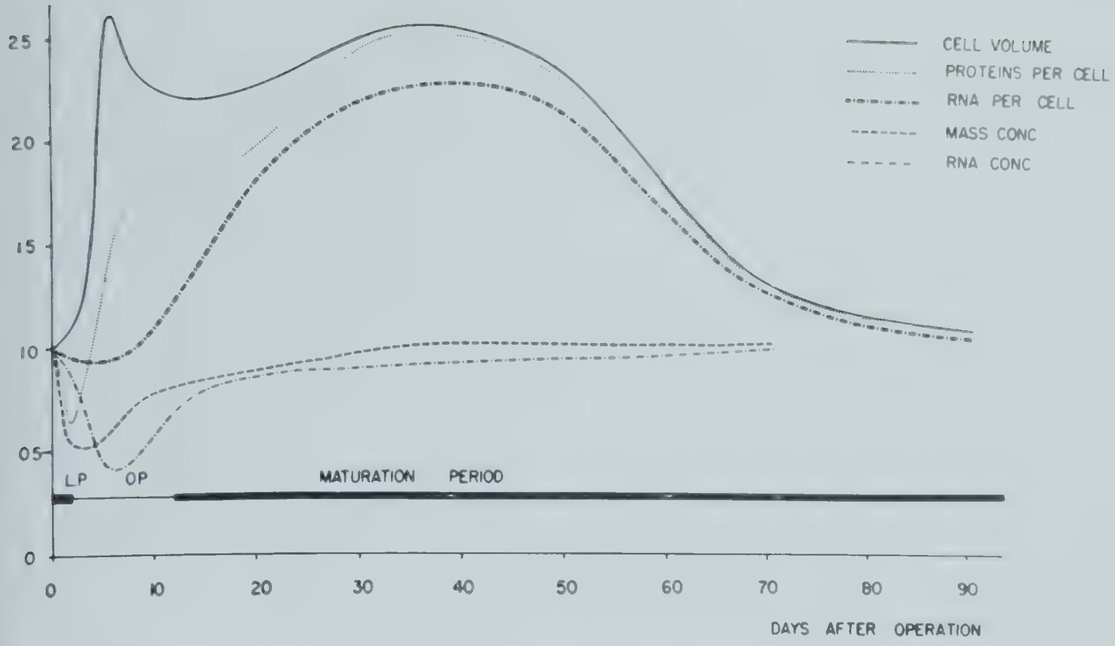


FIG. 17. Diagrammatic representation of the quantitative changes in hypoglossal cells after nerve crush (see text). From Brattgård *et al.* (1957).

The quantitative results of a study of regenerated hypoglossal nerve cells (Fig. 17) showed a decrease in the amount of proteins per cell of ~30% during the latent period. During the outgrowth of the axons there were several striking changes. There was a decrease in the RNA concentration per cell, confirming the results of the ultraviolet studies; but the amount of RNA per cell, 200 pg., remains unchanged. This was explained by a 260% volume increase of the nerve cell body due to an uptake of water. In spite of the decrease in protein concentration in the cell, when the protein amount was determined, an increase of 100% was found during the first two days; further protein increase resulted in a doubling of the amount. The amounts of lipids also increased, paralleling the proteins. Although the RNA remains quantitatively the same, there is a change in the aggregation of RNA toward a finely dispersed aggregation. This was assumed to mean a change of the RNA from a less active to a more active form in this stage of protein increase during dilution of the cell material. It explains the stained picture of the chromatolytic cell. The typical displacement of the nucleus may be explained by the changes in viscosity in connection with the concentration decrease and a speeded-up protein production directed toward and down the axon. The similarity with a glandular cell seems striking. A day after trauma, the synaptic endings were found to be swollen and the vesicles and mitochondria had degenerated or disappeared (De Robertis, 1956).

The finding of a nearly 100% increase of the mitochondria in regenerating hypoglossal cells during this period and electron microscopic changes is relevant (Hartmann, 1947, 1954, 1956). It may mean that the oxidative process in these nerve cells has been speeded up. There are also observations indicating an increased metabolism of proteins and RNA in regenerating nerve cells during the outgrowth period. Fischer *et al.* (1958) found that the cells incorporated per unit area more S^{35}-methionine than did the control cells. Brattgård and Hydén (1960) used C^{14}-orotic acid injected intracisternally and found that the regenerating hypoglossal cells incorporated per cell more of the labeled nucleic acid precursor than did the control cells. This confirms the conclusion that there is a high production and an increased metabolism of RNA and proteins in the nerve cells during the outgrowth of the axons. There seems to be no conduction impairment during retrograde chromatolysis, but a reduction of the action potential (Mark, 1949).

Thus the first changes are quite dramatic during the time when it is urgent for the nerve cell to spin out the thin axon to re-establish the functional contact with the periphery as soon as possible. The conditions in the degenerating peripheral part may be optimal also for regeneration during this time.

The maturation period. The changes now become different. The connection with the periphery has been re-established. It can be assumed that now the stimulus from the effector organ back to the cell which follows with the re-establishment of the functional contact, is the factor that changes the course of the chemical processes. Aitken (1949) found that the maturation of regenerating nerves is much more complete if the fibers make contact with the denervated muscle fibers. The RNA concentration has increased and the cells with the broad rings of RNA around the nuclei are most conspicuous (Fig. 18). Quantitatively there begins now a second period of volume increase which will become maximal about one month after the nerve crush. This cell growth is not, however, due to water uptake, but to a real increase in the amounts per cell of RNA, proteins, and lipids, which will be more than doubled. The RNA reaches values of 470 pg. per cell compared with 200 pg. for the control cell. The mass concentration has been restored by one month after the crush.

The Schwann cells undergo at that time changes which by analogy with other cells indicate a protein production. The nucleus increases enormously, the nucleolus enlarges, and RNA can be detected in the increased cytoplasm (Hydén and Rexed, 1943).

Conclusion. It may be said that the characteristic changes during the short outgrowth period may be regarded as the activation of an emergency reaction laid down in the nerve cell and resulting in a rapid production

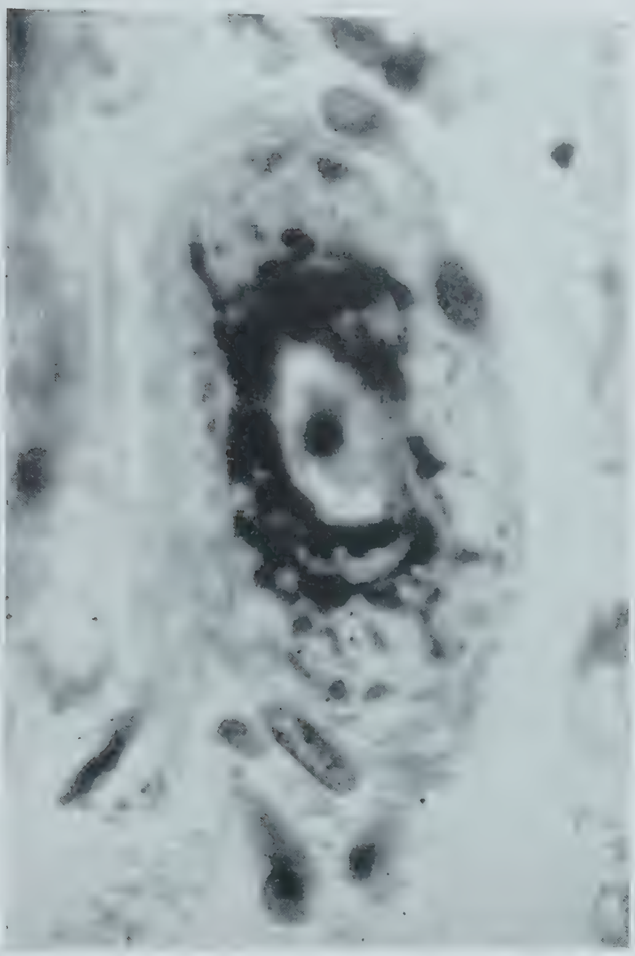


FIG. 18. Nerve cell from ganglion nodosum at 30 days after nerve crush photographed at 2570 A. Note the area containing RNA surrounding the nucleus. Magnification: ×1600. From Hydén (1943a).

of the new axon and its membrane. To determine whether other types of chromatolytic reaction also reflect a rapid production of proteins and an increased RNA metabolism under uptake of water, seems urgent. Therapeutic production of such a reaction may be a line of choice.

The maturation period, characterized by a volume increase of the nerve cell due to a large production of nucleoproteins and lipids, is initiated by the re-establishment of a functional relationship with the periphery. The making of this connection at the right time seems to be a most important factor and deserves further study.

V. AGING PROCESSES IN THE NEURON

Neurons cease early to divide and remain during the life cycle; thus, though other somatic cells may reconstitute tissue by mitosis, the neurons do not have this potentiality. There are many data on changes with age in the CNS. The conspicuous yellow pigment, lipofuscin, will be discussed below.

Bürger (1954) found that in man the brain loses approximately 100 gm. in weight during the lifetime. This is an involution that primarily strikes the pathways. Lipids and cholesterol increase, phosphatides and sulfatides decrease, with age. The absolute content of nitrogen of the rat brain was found to increase with age. The histidine concentration relative to that of arginine also increased (Clonet and Gaitonde, 1956). Weinbach and Garbus (1956) did not find any decline in O_2 consumption in phosphate uptake in brain mitochondria from old rats compared with those from young animals.

In the nerve cells in man, there emerge early in the postnatal period refracting granules in the cytoplasm that are stainable with osmium tetroxide. During the first decade these granules are faintly yellow and increase in amount. They are first detectable in anterior horn cells, in which they increase in amount and in color during the second decade so that they may fill up a part of, and even deform, the cell body. The amount of pigment decreases in the nerve cells going up in the medulla. The pigment can be seen both in sensory and motor cells, but is scanty in the Purkinje cells, even at advanced ages. It varies in amount in different parts of the CNS and has been found in man, primates, old cattle, and guinea pigs.

The pigment usually takes up a large and well-defined part of the cytoplasm, which may bulge and cause the nerve cell to appear pear-shaped. In earlier cytological studies, the pigment was described as being composed of a fraction stainable with lipid dyes which seems to be relatively richer at earlier ages. With increases in age, this fraction changes in color from light yellow to darker yellow. The pigment can be stained at pH greater than 5.5, where the Nssl substance does not stain. The isoelectric point has been determined to lie at pH 4.5. The pigment is very stable against organic solvents and acids (Pilcz, 1895; Mühlmann, 1900, 1901; Obersteiner, 1903; Oberndorfer, 1921; Volkmann, 1932; Zeglio, 1935; Bethe and Fluck, 1937; Altschul, 1938).

On the basis of these cytological observations, a general conception was that the lipofuscin represented a "wear and tear" substance. Altschul (1938), however, concluded that the pigment represented a supporting substance for nerve cells with decreasing capacity.

Dixon and Herbertson (1951) found glycolipids in this pigment which they considered to be aggregates derived from a more dispersed form of glycolipids resulting from cell traumata.

Nageotte (1907) considered that the yellow pigment was a derivative from the mitochondria, as did Hess (1955). The latter observed pictures in electron micrographs that indicated that the yellow pigment developed in relation to swollen and degenerated mitochondria.

Cytochemical studies have indicated with the possibility that the yellow pigment is a pteridine (Hydén and Lindström, 1950). Some new data have accumulated since then (Hydén, unpublished). In the region 2600–3000 A., the area containing pigment absorbs heavily and evenly in Carnoy-fixed nerve cell section. After removal of the RNA, strongly absorbing granules appear which were embedded in the RNA. The absorption spectrum before digestion of the RNA showed maxima at 2600 and 2800 A.; after removal of RNA there is a rather broad maximum at 2900–2950 A. The earlier report that treatment with ribonuclease did not remove the 2600 A. band (Hydén and Lindström, 1950) most certainly resulted from the inefficiency of the enzyme used. The fluorescence has been measured with a sensitive microfluorimeter (Bourghardt and Hydén, unpubl.). The emitted radiation has a sharp intensity maximum at 5000 A. and a small band at 4600 A. The presence of flavines can be excluded. The mass of the pigment seems to be about 50%, and the lipid content 30%, higher than that of the remainder of the cytoplasm. At microincineration the ash residue seems denser than in the remainder of the cell and is bluish-white in color in the dark field, compared with the yellow ash of the pigment-lacking parts. Valid determinations of iron in the pigment have not been carried out.

Judged by the cytological data and the UV spectrographic data on sections, it looks as if the amount of RNA decreases with increasing age when the amount of pigment increases. To settle this question microchemical RNA analyses were carried out on anterior horn cells from man—in all, 535 analyses from 41 persons (Hydén, unpublished). The average amount of RNA per nerve cell was found to increase from age 3 to age 40. It is then more or less constant up to age 55–60 and finally decreases significantly at higher ages. The amount of yellow pigment, determined by X-ray microradiography, increases up to age 40–50, although it varies in amount in the anterior horn cells and in different persons. But it does not decrease in amount at higher ages. Thus, there seems not to be an inverse correlation between the amount of RNA and yellow pigment per nerve cell.

Siebert *et al.* (1955) made a chemical elementary analysis of a purified suspension of lipofuscin granules and concluded that they contain insoluble, inactive proteins.

In conclusion, it can be said that the composition of the lipofuscin is still unsolved. It contains a lipoprotein with a complex chromophore, and nothing so far contradicts the working hypothesis that it belongs to the group of pteridines. As regards its function, it can be stressed that the pigment occurs physiologically and increases early with age, apparently without impairment of function, and in a type of cell which does not reconstitute itself chemically through mitosis. The lipofuscin may reflect a successively occurring chemical reconstitution of the nerve cell. It may take part actively in the cell metabolism.

VI. Thoughts on an Intracellular Mechanism Serving Memory

There are difficulties in formulating in unequivocal terms the problem of the memory mechanism in relation to the activities of the CNS. It can be phrased as learning, retention, and recalling at various levels, in relation to man in society, to the nerve tissue organization, the individual neuron or living protoplasm, or phrased as a molecular problem. It seems to encompass a variety of mechanisms in nature, if change by use is taken as a definition. The capacity to recall the past to consciousness can certainly be expected to reside in a primary mechanism of general validity in biology. The purpose of the following section is to discuss briefly the plausibility of a mechanism which is linked up with the genetic stability of cells as well as with the specific neuronal function. Function of chromosomes and genes can also be phrased in terms of "change by use" serving a memory mechanism.

A. *Earlier Views on Learning and Recall Mechanisms*

Some previous views on learning and recall mechanisms in the CNS were based on electrophysiological data; knowledge and assumptions on the properties of the synaptic knobs and the three-dimensional pattern of connections between neurons; and analogies with electronic computers. On the whole this discussion is centered rather on the spreading of impulses in a pattern than on intracellular events serving the mechanism. Eccles (1953) has worked out one of the most consistent recent hypotheses. The assumption of a change and a selectivity in the synaptic knobs or their membranes is involved here as well as in previous hypotheses worked out by, e.g., Konorsky (1950) and Hebb (1949). Both the outgrowth of new synaptic knobs, the growth of knobs, and the specialization of their membranes have been discussed. The observation at high resolution of the delicate extensions of the nerve terminals and their complicated relations to the glia membranes, necessitates, however, consideration in the formulation of future memory hypothesis. The reverberatory neuronal connections described by Lorente de Nó (1934) have played a great role. Lashley (1950) based his memory hypothesis on a constant reverberatory excitation. A most important observation of Lashley is that a particular memory is not stored in a single locus but has a multiple representation. The experiments of Lashley indicate that the same neurons which participate in the recall of a certain memory, also are involved in certain other permutations in thousands of other memories. Another pertinent fact is that extinction of the electrical activity of the cortex did not affect engrams (Gerard, 1955).

Eccles assumed that a memory of short duration could be established by repeated excitation of certain synapses which temporarily altered the spatial relationship of the specialized knobs to their responding post-synaptic membrane. These plastic changes at the synapses comprising a growth should lead to enhanced functional efficiency and to facilitation in conditioned reflexes. The engram should be a patterned association of neurones, the activity of which is affected by usage and maintained for a long period.

Pringle (1951) assumed that the learning capacity is based on the property of a chain of neurons of behaving as loosely coupled oscillators. Frequency changes in the direction of an increase could affect the state of all oscillators, each neuron influencing the next with a certain lag in oscillation. The rapidity in the learning process is consistent with the rapidity in a model based on complexity in time. The difficulty with significant morphological changes of the synapses is thus overcome. The memory of long duration could be explained as due to slight changes in a large number of nerve cells by which the excitation, in Pringle's meaning, reacted back on its internal chemical state on the molecular level.

These are a few points in this wide field; for a discussion see the Hixon Symposium (1951), Eccles (1953), Thorpe (1956), Burns (1958), Sperry (1955) and Young (1955).

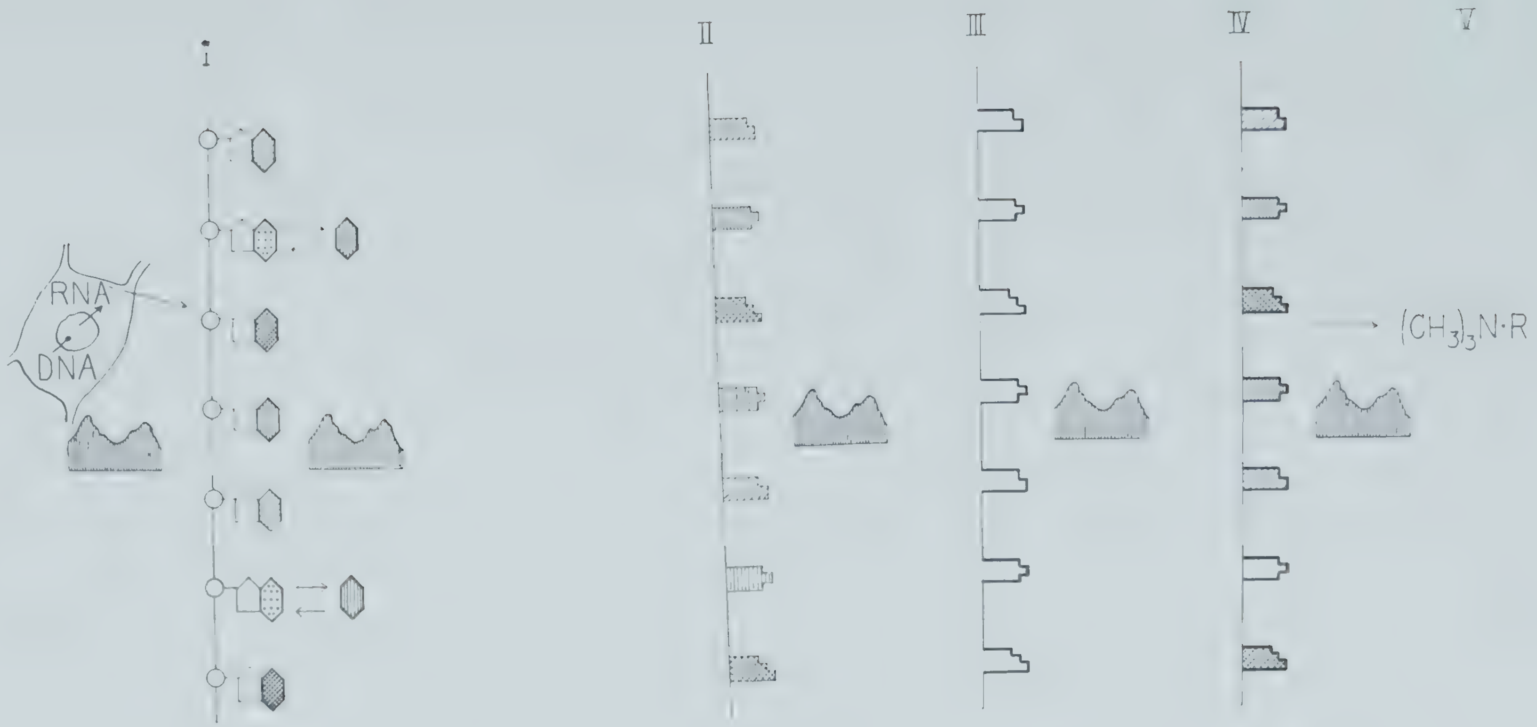
B. *A New Hypothesis*

The following view may be food for thought for further discussion of an intracellular mechanism for the storage of information in the form of molecular specificity in the neurons and for the selectivity in its operation. It is based on the fact that nucleoproteins are produced at increasing neuronal activity and on the assumption that this protein production is as characteristic for the neuron as is the impulse production and propagation and that the proteins produced may be specific. The three-dimensional pattern of neurons is developed early, and factors reflecting the biochemical specificity of different neurons and the membrane properties can be assumed to play a role in the formation of connections (Hamburger, 1955).

In view of the current knowledge of the formation of cell constituents, DNA is a dominating molecule conducting the synthesizing processes in the cell with respect to genetic specificity. The DNA of one species differs in certain basic properties from that of another species. It seems that the DNA controls the production of cytoplasmic proteins through the mediation of RNA. That implies that the RNA molecule is the template structure which directly determines the specificity of the proteins formed, but that RNA is itself specified as to its general structure by DNA and gene action. From the findings of Allfrey *et al.* (1955, 1957) and associates, Zamecnik and Keller (1954), Littlefield and Keller (1957), Hoagland *et al.* (1957), and particularly those of Novelli and De Moss (1957) and Konigsberger *et al.* (1957), a tentative scheme of cytoplasmic protein production can be visualized. Proteins are formed directly from free amino acids. The initiating step is amino acid activation by enzymes through the carboxyl groups, ATP furnishing the energy. The activated amino acids are transferred to RNA, where a bond between the carboxyl group of the amino acid and RNA is established. Finally, there is a condensation of the amino acids to a polypeptide polymer. The sequence of the bases in the RNA molecule may be the principle according to which the crucial and active part of the protein molecule is formed, thus determining its amino acid sequence. Rich (1958) has analyzed the structure of synthetic polyribonucleotides and has found that they can form hydrogen-bonded, two-stranded helical structures, as does most probably also the naturally occurring RNA. Novelli and DeMoss (1957) argue that the RNA alone cannot act as a template structure. The activated amino acids would come too far apart for a condensation reaction to occur. Therefore, they consider it more likely that the organizer system for protein synthesis is a ribonucleoprotein. For the following thought experiment this of no importance, as will be pointed out below.

The next factor to consider is the nature of the signals recorded from the neurons. Most probably this code can be considered as a frequency modulation (Taylor, 1957).

The first step in the intracellular mechanism for producing an engram can be visualized as a specification of RNA in the nerve cell by the first series of impulses generated in sensory cells or in motor neurons pre- or postnatally (Fig. 19). In the case of the motor neurons it is a mutual action between the activity of the muscle fibers and their neurons. The modulated frequency as an electrical pattern can be thought to affect the ionic equilibrium in the cytoplasm. This changes the stability of one or more of the four bases of the RNA molecule at a certain site, depending on the modulated frequency (I in Fig. 19). The effect of this is an exchange of one against another base from the surrounding pool. The new base fits the space now available in the molecule and is stable under the influence of this modulated frequency. The effect of the first series of



DNA of the species directs on a long-term **basis the protein production in** the cytoplasm **via the RNA.**

RNA Specification through modulated **frequencies.**

produces

Protein specified

which becomes activated by combination with a complementary **molecule and**

releases transmitter substance which excites the post- **synaptic cell.**

Fig. 19. Diagram of the changes discussed in the hypothesis of a memory mechanism.

excitations is thus the above-mentioned *specification of the RNA in the nerve cell* by a change in the sequence of bases. The new distribution pattern is then supposed to remain.

The second step in the process follows from this specification of the RNA molecule. At increased neuronal activity RNA and protein are formed in the cell (see page 274). Since the sequence of the bases in the RNA is now changed, the protein formed through the mediation of this RNA will also be specified (II in Fig. 19). The importance of the amino acid sequence for the activity of the protein has been amply shown. Thus, the amino acid sequences of oxytocin and vasopressin differ only in two residues (Popenoe and du Vigneaud, 1954). Considering information transfer from nucleic acid to protein, Gamow *et al.* (1956) have discussed the possible arrangement of bases in the polynucleotide chain, to which there corresponds a unique arrangement of amino acids in the polypeptide chain synthesized by RNA. The mechanism could be one where a sequence using four symbols transfers molecular specificity to another comprising twenty symbols. If the determining code consisted of three successive pairs of bases in the RNA chain, a triplet arranged in a diamond form, such a triplet could determine the site of one amino acid. Also other types of models were considered. In statistical testing of the amino acid distribution in a sequence of known proteins, the results indicated a random distribution of amino acids. There is a probability that a triplet of bases randomly distributed along the RNA chain determines the amino acid sequence in a protein.

Whatever the mechanism, the second step in this memory scheme comprises production of a specified protein resulting from a change in the RNA molecule which was the result of the first series of the modulated frequencies going through the neuron as impulses.

The third step can be assumed to consist in a *rapid dissociation* of the specified protein and the combination of the dissociated products with a complementary molecule or an energy activation of the specified dissociated protein (III and IV in Fig. 19). The nerve tissue has a capacity to respond rapidly with a production of nucleoproteins and their split products. Geiger *et al.* (1956) found that stimulation for 30 seconds was sufficient to produce nucleic acid split products of basic character. Through a rapid combination of the dissociated protein with a complementary molecule in the third step, an activation then occurs of the transmitter substance and the postsynaptic structure is excited in the fourth step of this assumed process (IV in Fig. 19). The transmitter substance may only facilitate the modulated frequency entering the postsynaptic area having a more informative character. A few types of transmitter substances may therefore be sufficient.

If a ribonucleoprotein as a complex should act as the template structure, as suggested by Novelli and DeMoss (1957), this would occur in the suggested scheme if the RNA in step II and the protein in step III acted together as a complex.

C. *Discussion and Summary*

The mechanism thus operates as follows. The neurons are interconnected in the most complicated chains. When the neurons in an already established memory trace are activated, regardless of how many they are or where they are located, the hypothesis implies the reaction to comprise the rapid *third and fourth steps* in the scheme. The impulses in the form of modulated frequencies activates the specified protein which releases the transmitter substance exciting the postsynaptic structure. The transmitter substance may be acetylcholine or any assumed inhibitory substance. The informative signal may, however, be the modulated frequency.

The reason why just this neuron responds, and all the others belonging to this chain, is that the protein which had once been specified by a modulated frequency now responds to the same type of electrical pattern whenever it is repeated. The RNA which was likewise specified in the sequences of bases by the exchange reaction, is produced and produces specified protein steadily, as a result of the neuron's activity. But the activation of the protein leading to the transmitter explosion occurs only at excitation. This mechanism would explain why a certain chain of neurons is excited by one stimulus, but rejects another. The number of permutations that can arise in the RNA molecules by alterations in the sequences of the bases is very great. It could easily constitute the storage of 10^{15} informations, which is a probable number for a man during 70 years of life.

Such a substrate for the learning and recalling mechanism would not require a strict localization within the CNS. The same neuron could participate as a link in many neuronal nets. Whether it did would depend on the response of the sequence of bases in the RNA to the modulated frequencies. By the successive specification of the neurons, association pathways could grow out, complicated in form and functioning after a basically simple principle.

With increasing years permutations with more statistical weight in the RNA molecule could be engaged in increasing numbers, leaving for new memory combinations statistically more improbable permutations. This could explain the characteristics of the learning and recalling capacity of old persons.

This hypothesis on the intraneuronal mechanism for memory has its anchorage in the genetic mechanism and assumes the substrate for memory to consist of the types of molecules which play the most important role in this primary biological reaction. It also provides a tentative explanation of part of the nucleoprotein production in the neuron, which should thus meet the steady need for specific proteins serving the neuronal function.

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CHAPTER 6

Visual Photoreceptor Structures

By WILLIAM H. MILLER

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I. Introduction

The ability to detect and react to light is an almost universal trait of living organisms. The ability to sense light is, of course, dependent on the light being absorbed and exciting a receptor. In animals that have eyes, the active light-absorbing substances are located within visual receptor cells and termed *visual pigments*. Such substances are not dispersed throughout the visual receptor cells. It is known in the case of some eyes, and strongly suspected of many others, that the visual pigments are concentrated in specially differentiated organelles of the visual cells, the photoreceptor structures. As is the case in other areas of biology, our knowledge of these structures is derived from several avenues of approach. From the microscopist's art we learn the morphology of photoreceptors. Much of this knowledge, particularly that relating to the vertebrates, is brought together in reviews by Arey (1932), Kolmer (1936), Polyak (1941), Walls (1942), and Detwiler (1943). Chemistry is another technique to which we owe our knowledge of photoreceptors. The large body of knowledge of the biochemistry of visual pigments is summarized by Dartnall (1957). Both microscopy and chemistry are sciences whose results are usually based on analysis of fixed or extracted components. A third main avenue of approach to photoreceptors is the study of the activity of the living cell. The reader will find an excellent treatment of this subject in Granit's book (1955).

It is the aim of this chapter to discuss for photoreceptor structures what seem to be significant features of each of the three aforementioned approaches. The morphology section of this chapter stresses results gained by the technique of electron microscopy on both vertebrate and invertebrate material. In the biochemistry section an effort is made to outline recent findings that relate to the mechanism of photoreceptor activity. In the section on physiology, as well as in the other sections, the discussion is restricted as closely as possible to direct evidence which bears on photoreceptor structures.

II. Photoreceptor Structure

A. *Vertebrate Rod and Cone Outer Segments*

The vertebrate eye is an extraordinarily complex organ, and it is, therefore, not surprising that the functions of all of its parts were not correctly deduced by early investigators. According to Polyak (1941), although the ancient Greeks had a good understanding of the gross anatomy of vertebrate eyes, they supposed the crystalline lens to be the organ of photoreception. This misconception persisted until studies of the microscopic structure of the retina showed that the rod and cone cells are the initial elements of the optic pathway. Because of this anatomical position, the function of the rods and cones as photoreceptors was postulated from the time of their discovery. Further, on the basis of careful observations of their distribution in the retinas of man and lower vertebrates, Max Schultze in 1866 postulated that the rods and cones subserve different percipient functions. The rods, which thickly populate man's peripheral retina and the entire retina in many nocturnal vertebrates, mediate vision at low levels of illumination. The cones, which are prominent in the retinas of diurnal vertebrates and predominant in the central fovea, are responsible for daylight vision and color sensations (see Hecht's review, 1937). That the rods and cones are photoreceptors is now firmly established by the knowledge of the presence

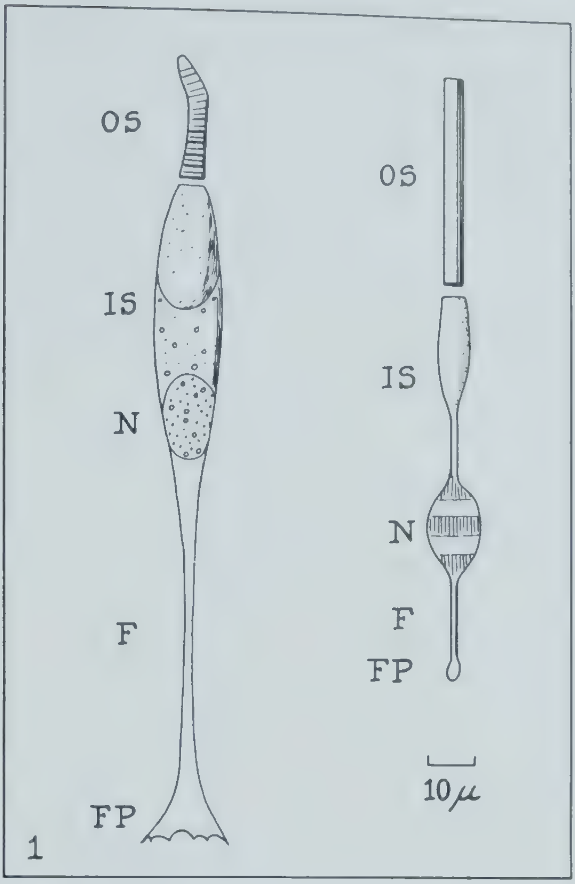

FIG. 1. Examples of vertebrate visual receptor cells. Left, macaque cone. Right, guinea pig rod. General regions of each cell are indicated: outer segment (*OS*), inner segment (*IS*), nucleus (*N*), fiber (*F*), and foot piece (*FP*). The separation indicated between the outer and inner segments is an artifact; they are actually connected by a thin stalk, the "connecting cilium" (see Fig. 6). From Schultze (1867).

of visual pigments in the outer segments of these cells (see Sections III and IV). The rods and cones (Fig. 1, from Schultze, 1867) are rather long, thin cells that differ from one another in shape, but in some cases it is very difficult to distinguish these cells on morphological grounds. Their morphology varies not only from animal to animal, but also from region to region of the retina. It is of interest that in the case of the cones, their shape is, according to O'Brien (1946), responsible for the directional sensitivity of these cells that was discovered by Stiles and Crawford (1933) and Stiles (1939). At their most vitreal ends, the rods and cones (Fig. 1) have a specialized synaptic region, the foot piece (*FP*), which is connected to the region of the nucleus (*N*) by a fiber (*F*). Next to the nucleus is the inner segment, which is sometimes contractile and often contains colored oil droplets and other specialized organelles. At the scleral pole of each cell is the outer segment (*OS*), which is typically cylindrical in the rods and conical in the cones; and since the outer segments are the actual photoreceptor structures, this discussion will be confined to these organelles.

1. Morphology

One of the earliest successful investigations of the morphology of outer segments was accomplished by Max Schultze (1866, 1867; and 1879 in English). Working with the retinas of animals from all classes of vertebrates he found that when examined in the fresh state, both rod and cone outer segments show "a very apparent and regular striation [which] leads quickly to a separation of the substance into disks." Using material preserved by means of a fixative which recent-day microscopists have found most satisfactory in studying fine structure, Schultze confirmed these observations: "The external segment of the rods of Man and Mammals which whilst still warm have been placed in a one or two per cent solution of perosmic acid, and have been thus preserved absolutely unaltered in form, exhibit, when examined with a power magnifying 1000 diameters and very oblique illumination, a striation which is as sharp as a hair drawn on copper. . . . This would correspond to a distance between the lines of 0.3 to 0.4 of a micromillimeter. In the cones the disks are somewhat thicker." Schultze continues, "Fresh rods [outer segments] examined in serum exhibit here and there longitudinal fissures. . . . I have shown that the longitudinal striation, which is more easily seen in Amphibia and Fishes on account of the larger size of the outer segments, is also present in Man and Mammals, and here also probably depends upon a channelling of the surface. It is a highly remarkable circumstance that the transverse section of the large external segments of Amphibia (Triton) and Fish (Syngnathus) often differs considerably from the circular form, and may present an irregular dentated border, or even be of semilunar shape."

There has not been an opportunity until very recently to validate directly Schultze's observations, because the structures involved can just barely be resolved by the light microscope. Indirect evidence of lamellation obtained by investigation of the birefringence of fresh and fixed outer segments using the polarizing microscope is reviewed by W. J. Schmidt (1951), who has done much of this work. Schmidt concludes that the rod outer segment is composed of alternating transverse layers of lipid and protein, the lipid molecules being oriented parallel with, and the protein molecules perpendicular to, the long axis of the rod. Direct evidence, however, has been obtained by the use of the electron microscope which has a resolving power roughly 100 times that of the light microscope. Though it is impossible to make observations on living material with this instrument, there is much evidence to suggest that recent techniques succeed remarkably well in showing structure as it is in the living cell (Porter, 1953; Palade and Porter, 1954; Müller, 1957; Sjöstrand and Baker, 1958). In a series of careful studies using the electron microscope, F. S. Sjöstrand has clearly demonstrated the existence of a lamellar structure in vertebrate rod and cone outer segments. The first electron micrographs were of osmium-fixed guinea pig rod outer segments which had been fragmented by sonic treatment (Sjöstrand, 1949). This study showed disklike structural units which are lobular, having an incision toward the center which accounts for the appearance of longitudinal grooving that Schultze commented upon. A clearer conception of the fine structure is obtained from electron micrographs of thin sections of outer segments (Sjöstrand, 1953, 1956, 1959a, b; Sjöstrand and Elfvin, 1957). The lamellar nature of rod outer segments as seen in longitudinal section is demonstrated by Fig. 2, one of Sjöstrand's electron micrographs of a fish (perch) rod. Higher magnification electron micrographs of thin sections of rod and cone outer segments show that their disklike structural units are not solid; for example, in longitudinal section, guinea pig rod disks consist of a continuous dense outline 30 A. thick (interpreted as a membrane) and an enclosed space 70 A. in depth occupied by a less dense material. Thus the total thickness of a single guinea pig rod disk is about 130 A. Similarly Sjöstrand finds the thickness of a perch rod disk to be 110 A., and that of the toad *Bufo bufo*, 110 A. The spacing of disks is 190 A. for the perch and toad and 240–340 A. for the guinea pig. Figure 3 is a high-resolution micrograph by Sjöstrand of a perch rod outer segment in longitudinal section.

The cone outer segments are very similar to those of the rods, as can be seen in Fig. 4, Sjöstrand's electron micrograph of a perch cone outer segment in longitudinal section. Sjöstrand finds that the thickness of cone disks varies from 100 to 170 A. In fact, the dimensions of both the rod and cone disks and their spacing vary according to the procedures of fixation and embedding, and these measurements therefore may not correspond exactly to those in the living state. The stack of disks in both rod and cone outer segments is enclosed by the plasma membrane of the visual cell. In some receptors Sjöstrand finds that the disks are connected to one another by tubulelike structures. In others, such as the two twin-cone outer segments depicted in Fig. 5, he has found evidence that all of the disks of an outer segment are formed of "a continuous membrane that is repeatedly folded" (Sjöstrand, 1959b).

The main morphological feature of vertebrate photoreceptors—a layered lamellar structure—has been found in a number of vertebrate eyes which have been examined by means of the electron microscope: an amphibian, the frog (Porter, 1957; Yamada, 1957); a reptile, the gecko

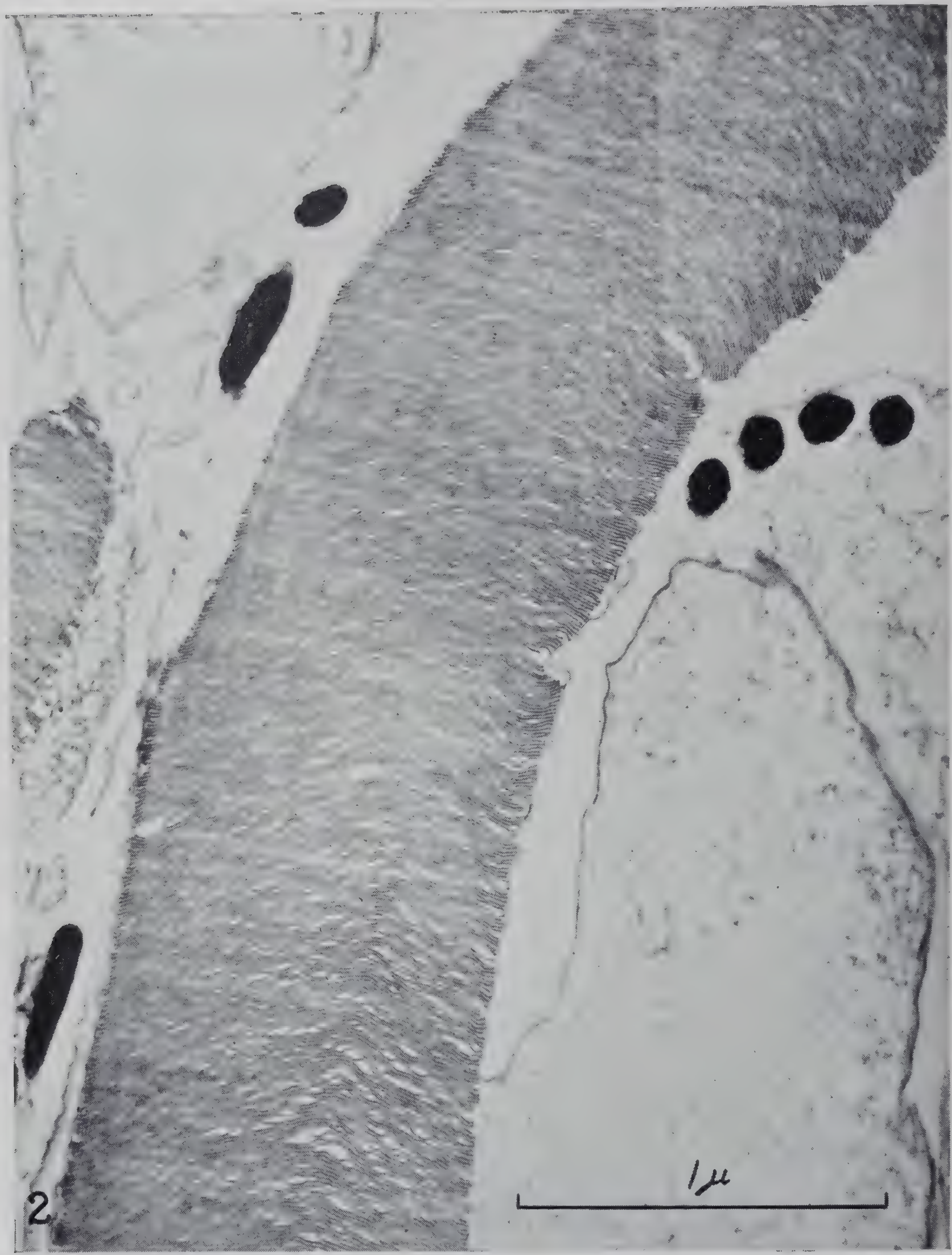

FIG. 2. Electron micrograph of a rod outer segment from a perch eye in longitudinal section, showing lamellar structure. From Sjöstrand (1959b).

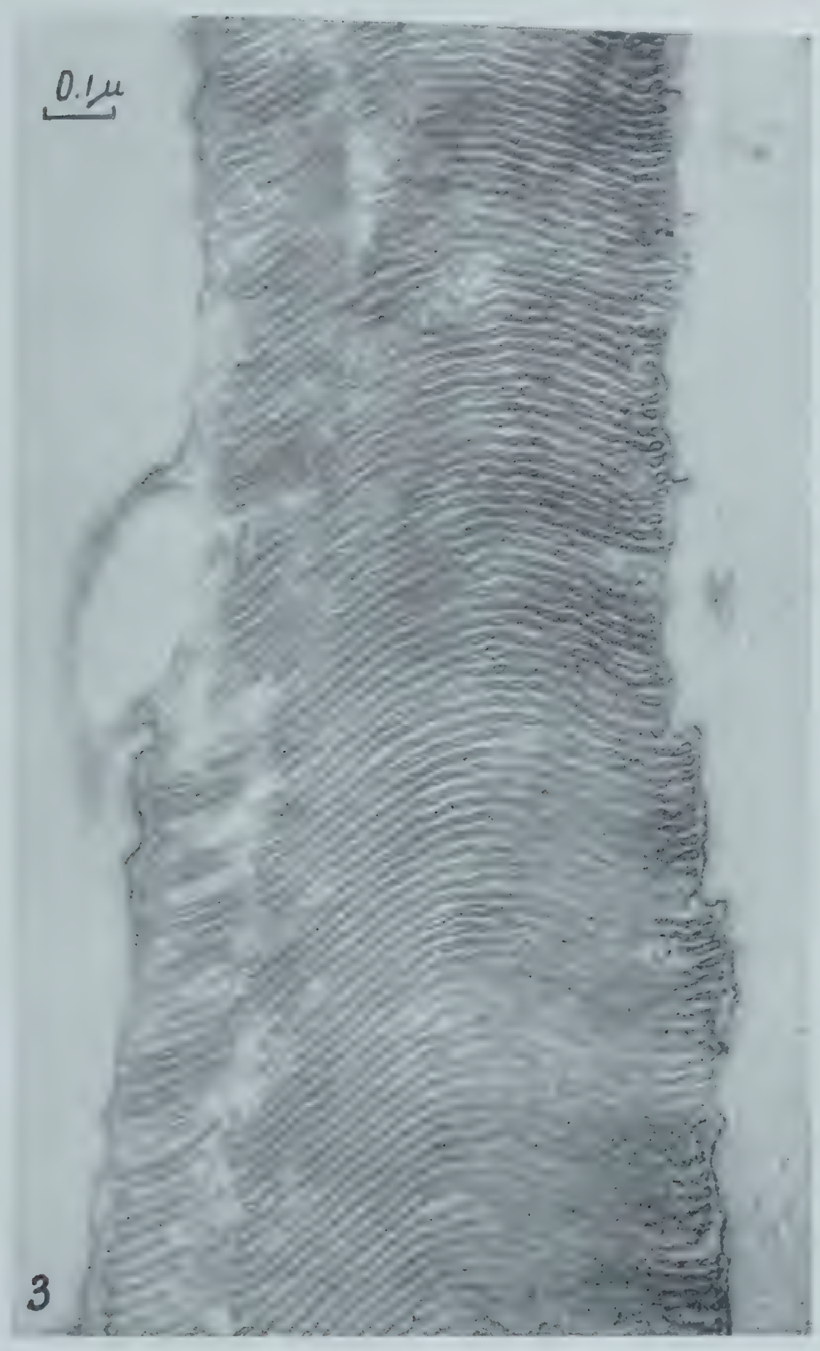

FIG. 3. High magnification electron micrograph of a perch rod outer segment in longitudinal section. From Sjöstrand (1959a).

(Carasso, 1957); and mammals, man (Yamada *et al.*, 1958), the rabbit (De Robertis, 1956a), and the mouse (De Robertis, 1956b).

2. *Embryology*

Careful light microscopy has indicated that the rods and cones are derived from ependyma, the flagellated epithelium that lines the neural tube and optic cup, and that the rod and cone outer segments are rooted

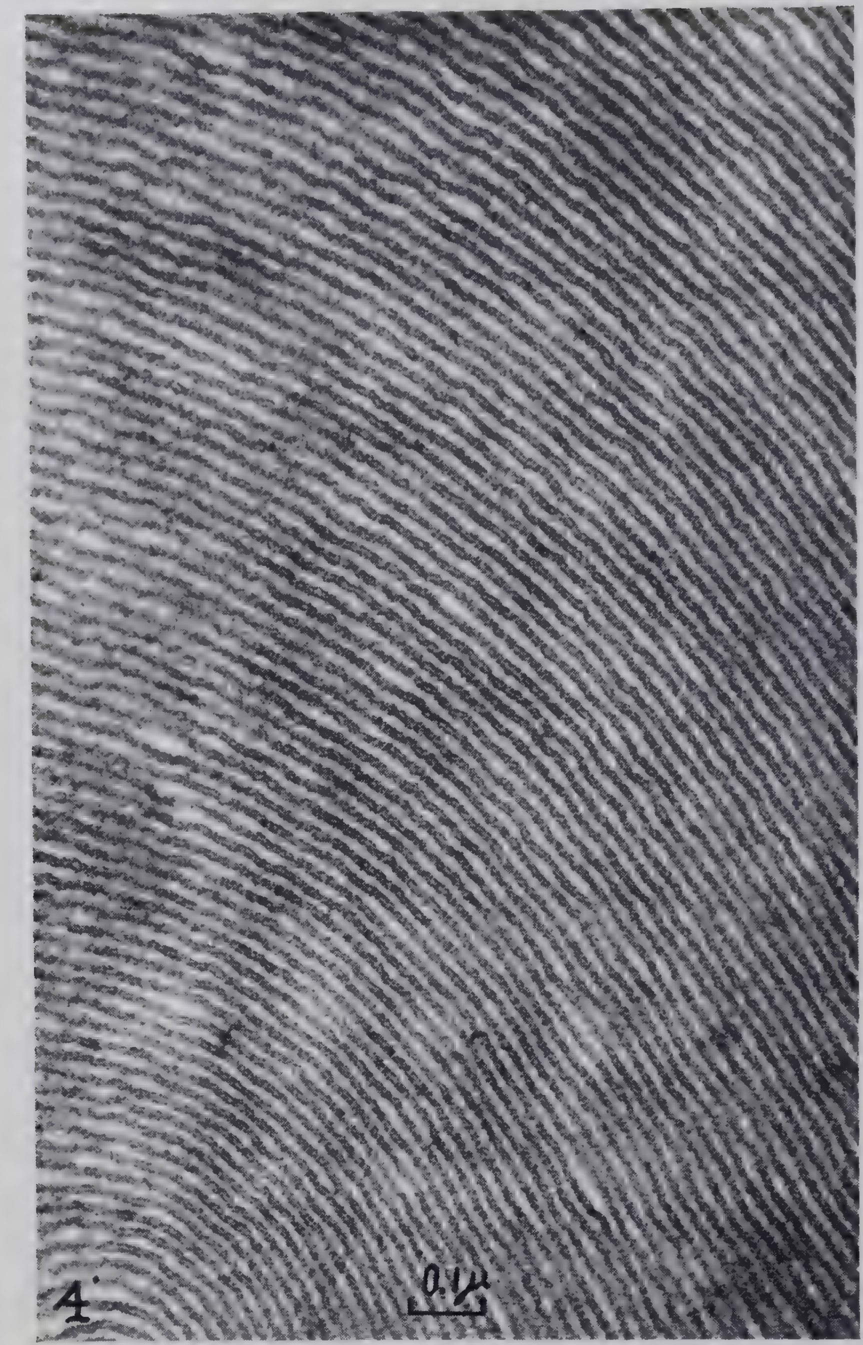

FIG. 4. High magnification electron micrograph of a perch cone outer segment in longitudinal section. From Sjöstrand (1959a).

in diplosomes and are therefore homologous to flagella (Mann, 1928; Kolmer, 1936; Walls, 1939). Just as was the case with the morphological findings of the fully developed outer segments, here again the electron microscope confirms and extends these light microscope observations. De Robertis (1956a) has found that rod outer and inner segments in the rabbit retina are connected by a ciliumlike structure, which he calls a "connecting cilium," containing nine peripheral double filaments as well

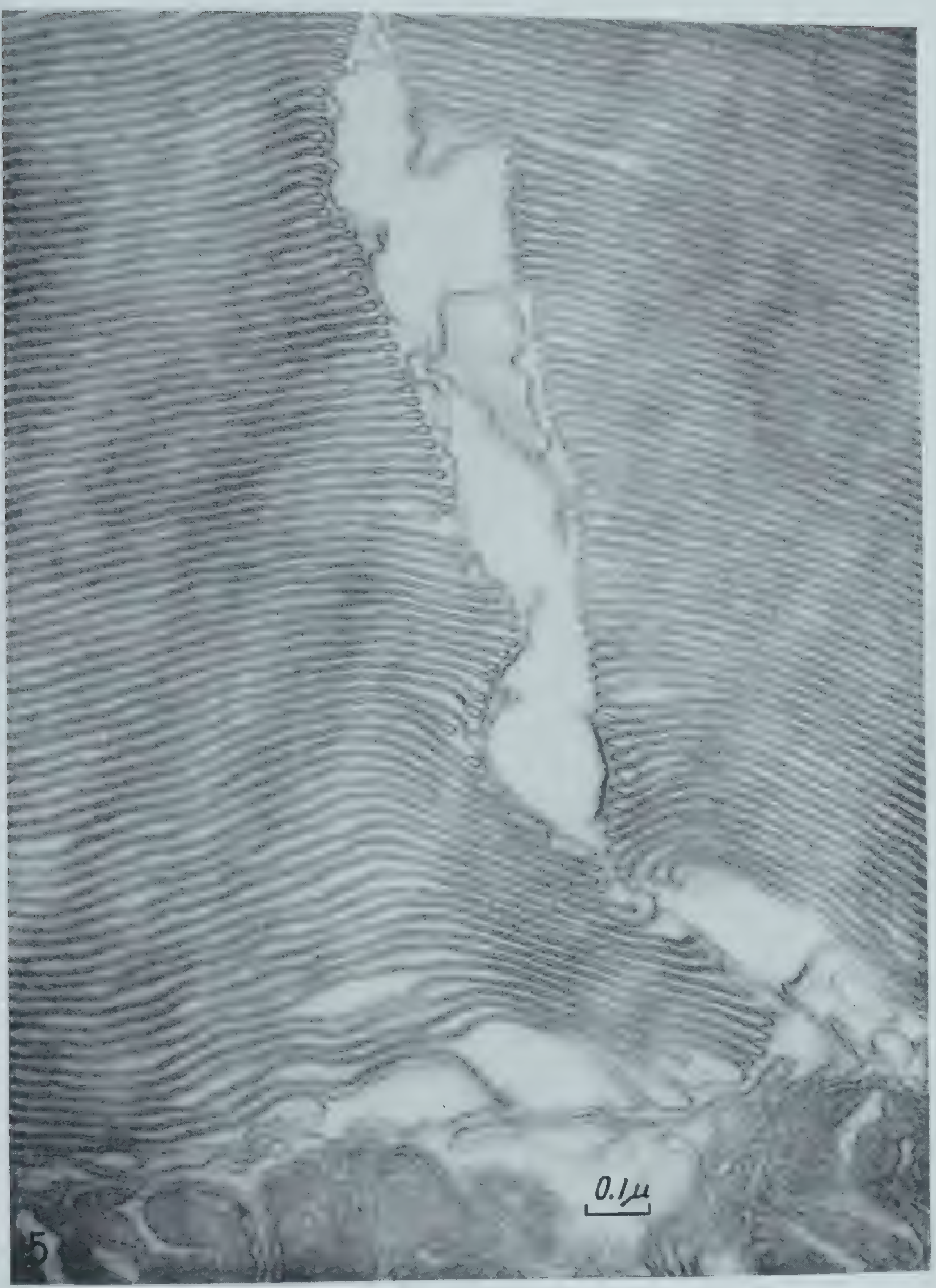

FIG. 5. Electron micrograph of parts of the outer segments of two twin cones of the perch retina in longitudinal section. Sjöstrand has found that the lamellar structure of these outer segments is formed of one repeatedly folded membrane. From Sjöstrand (1959b).

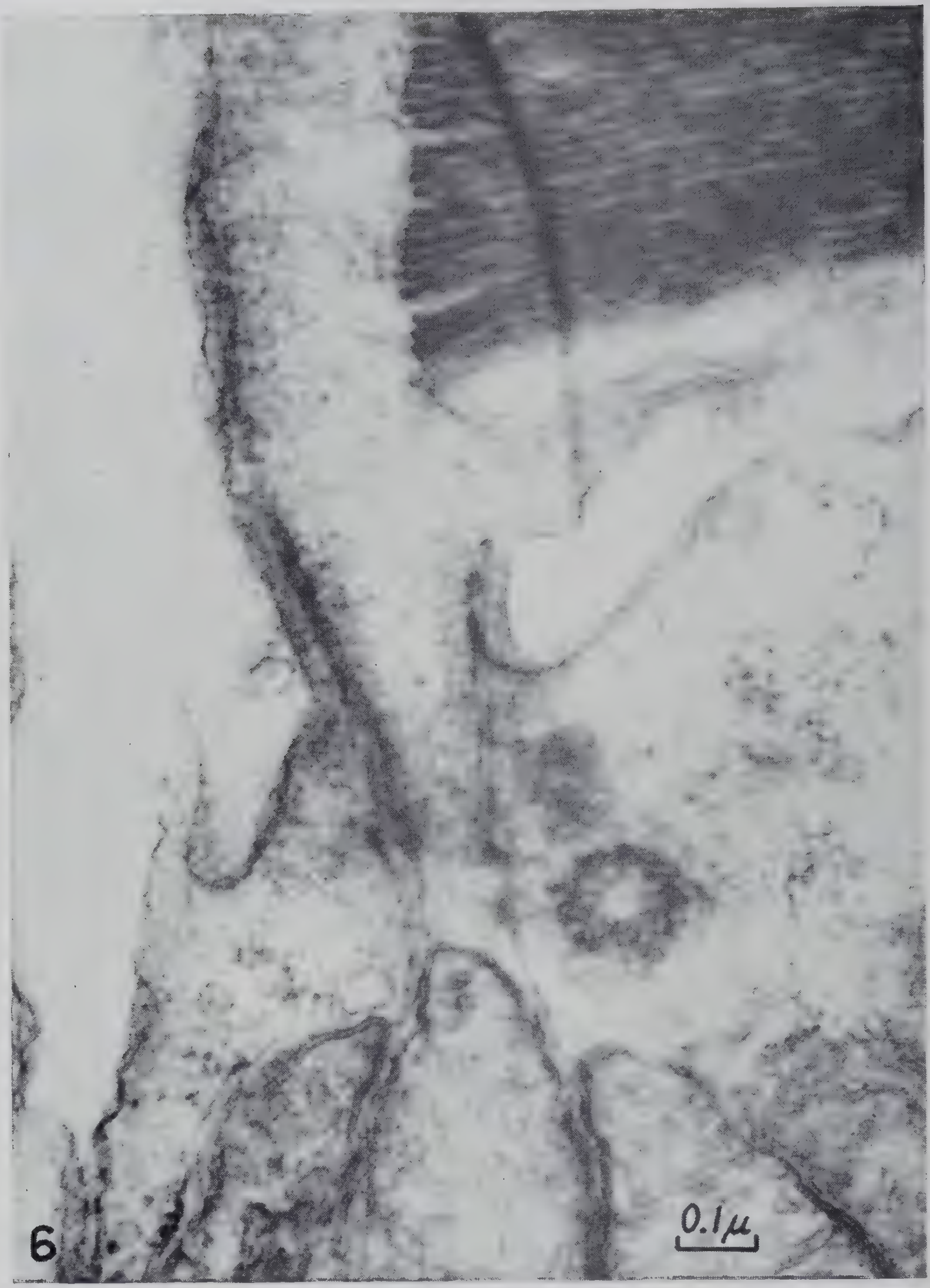

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Fig. 6. Electron micrograph of connecting cilium in a rod of the frog retina. The lamellae of the outer segment are seen in the upper right of the figure. A few filaments are seen in the connecting cilium which ends just above a cluster of mitochondria in the inner segment. Just to the right of the base of the connecting cilium is an accessory basal body in cross section. Unpublished micrograph of Dr. Yamada, Rockefeller Institute, New York.

as occasionally a central pair. Figure 6 is an electron micrograph by Dr. E. Yamada of the Rockefeller Institute which shows part of a frog rod outer segment, inner segment, and the connecting cilium. In the inner segment near and at right angles to the basal body of the connecting cilium, an accessory diplosome is seen. Connecting cilia have also been observed in electron micrographs of the rat rod (Porter, 1957) and the toad cone (Sjöstrand and Elfvin, 1957). In the mouse retina the development of the rod outer segments is not completed until after birth. By investigating the morphogenesis of the outer segment from birth to the sixteenth day, De Robertis (1956b) traced the development from the stage at which the primordial outer segment consisted of only a "primitive cilium" to the fully differentiated adult rod. The disklike structural units of the outer segments appeared to develop from the fine vesicular matrix of the primitive cilium. In a more recent study by Tokuyasu and Yamada (1959) the disks of kitten rod outer segments are observed to develop from tubular invaginations of the plasma membrane of the outer segment which are in close association with the ciliary filaments; and it is concluded that vertebrate photoreceptors are clearly ciliary derivatives.

B. Invertebrate Photoreceptors

Some animals in most of the invertebrate phyla have what one might call photoreceptors, but well-developed eyes are for the most part confined to the mollusks, arthropods, and annelids (see Milne and Milne, 1956, for bibliography). Eyes of animals of these phyla usually contain a dioptric system and a retina in which there are cells, which, because they are the initial elements of the neural chain that leads to the animal's central nervous system, are considered to be visual receptor cells. And these cells, like the vertebrate rods and cones, have highly refractile parts which are named according to their shape, e.g., "mantel" or "rod" ("rhabdomeres" which comprise the "rhabdom" in the arthropod's ommatidium), and which, like the vertebrate outer segments, have generally been assumed to be the actual photoreceptor structure (Schultze, 1868; Grenacher, 1879; Parker, 1897; Hesse, 1907). This opinion has received support from recent evidence that a visual pigment is concentrated in the rhabdomeres of the lobster compound eye (Wald and Hubbard, 1957). The birefringent organelles of invertebrate receptor cells, like vertebrate outer segments, have a faintly striated appearance when viewed by the light microscope, a fact which led Hesse (1907) to call them *Stiftchensäume* (little pin borders).

1. Arthropods

The nature of the arthropod rhabdomere has been a major concern of students of invertebrate photoreceptors. Grenacher (1879) and Watase (1890) thought the rhabdomere to be a cuticular secretion, while Hesse (1907), Demoll (1914), and Parker (1897) believed it to be actually a part and substance of the receptor cell composed of neurofibrils. The problem has been clarified by electron microscope investigation of the rhabdomeres in the eyes of animals belonging to four classes of arthropods (Fernández-Morán, 1956, 1958; Miller, 1957a, b, 1958a; Goldsmith and Philpott, 1957; Wolken *et al.*, 1957; Danneel and Zeutzschel, 1957; Yasuzumi and Deguchi, 1958). These studies indicate that the structural unit of the arthropod rhabdomere is tubular and that the rhabdomere resembles a honeycomb on a small scale. The tubular units of the rhabdomere vary from 40 to 120 mμ in diameter depending on the animal and the fixation techniques. The arthropod rhabdomere may be thought of as being composed of tightly packed microvilli of the receptor cell's surface. This interpretation is illustrated by Fig. 7, an electron micrograph showing parts of three receptor cells of a spider eye sectioned parallel with the cornea. The division between receptor cells appears as a slight separation of the rhabdomeres. The rhabdomeres themselves resemble brush borders with the long axes of the microvilli predominating in the plane of the section, though the faint outlines of microvilli caught in oblique section are seen in parts of the section. The appearance of obliquely sectioned microvilli is illustrated in Fig. 8, an electron micrograph of a rhabdomere from the compound eye of a beetle, *Tenebrio molitor*. In a recent monograph on the compound eyes of insects, Fernández-Morán (1958) has high-resolution electron micrographs of insect rhabdoms. Figure 9 is a micrograph from that paper showing the appearance of the microvilli of a housefly rhabdomere as they appear in cross section.

The compound eye of the horseshoe crab, *Limulus*, is of particular interest because of our knowledge of its functional properties (see Section IV). This eye has roughly 800 ommatidia, the sensory portions of which consist of about a dozen wedge-shaped retinular cells arranged radially about the dendrite of a bipolar neuron, the eccentric cell (Watase, 1890; Demoll, 1914; Hartline *et al.*, 1952; Waterman and Wiersma, 1954; Miller, 1957a, 1958a). Figure 10 is a low magnification electron micrograph of a part of the sensory portion of an ommatidium in cross section. At the top of the figure is the dendritic distal process (*D*) of the eccentric cell. The dense circle around the distal process and the spokelike markings comprise the rhabdom, which is made up of the V-shaped contribution of each retinuclar cell (*RC*). Electron micrographs of higher magnification show that the rhabdomeres are composed of closely packed microvilli of the cell's surface. These microvilli as seen in longitudinal section are shown in Fig. 11, a. The arrows mark the plane of abutment of the

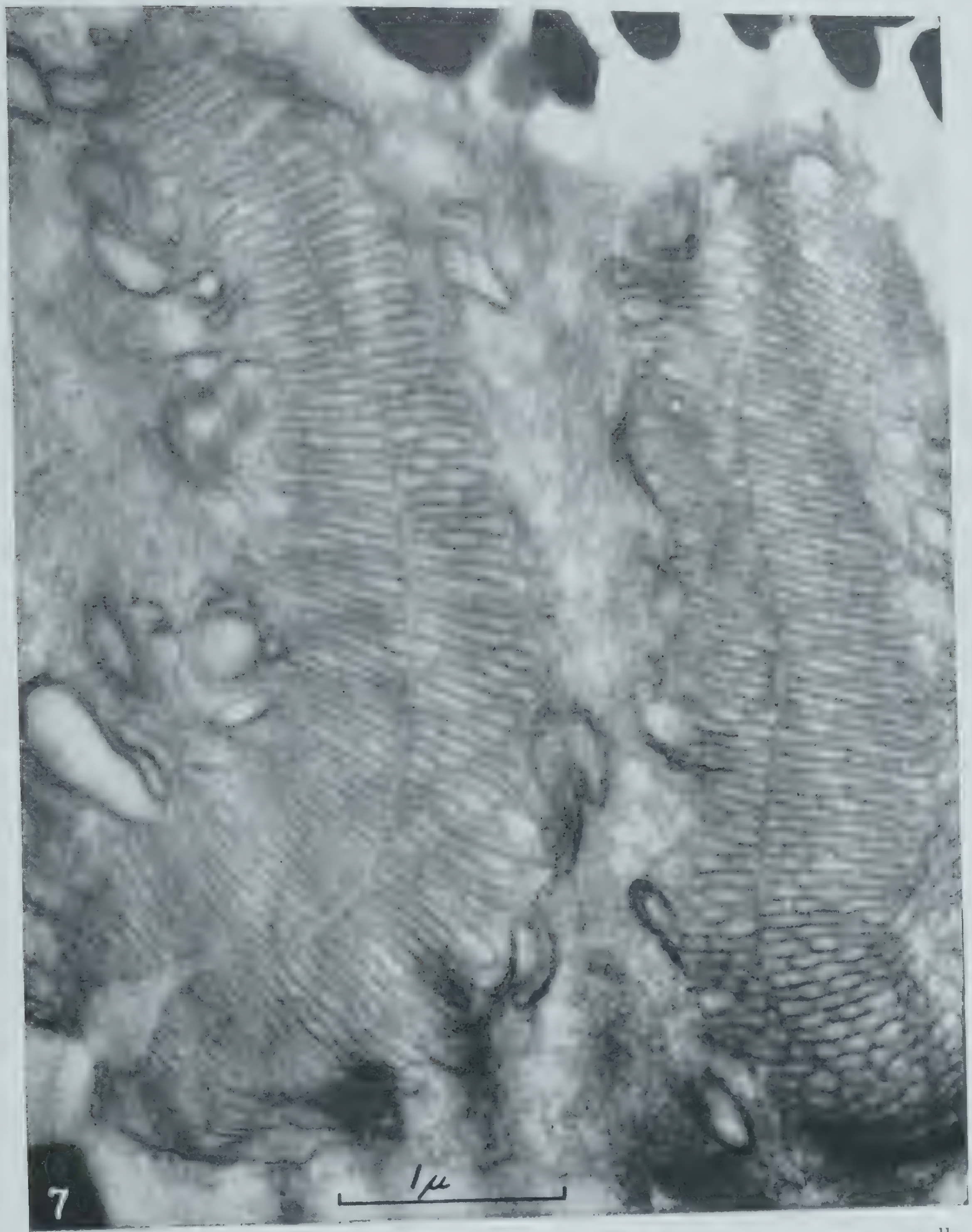

FIG. 7. Electron micrograph of parts of three spider visual receptor cells sectioned in a plane parallel to the cornea. The rhabdomeres of the cells consist of closely packed microvilli of the receptor cells' surfaces and are seen to resemble brush borders. The slight separation between rhabdomeres marks the plane of separation between contiguous receptor cells. From Miller (1957a).

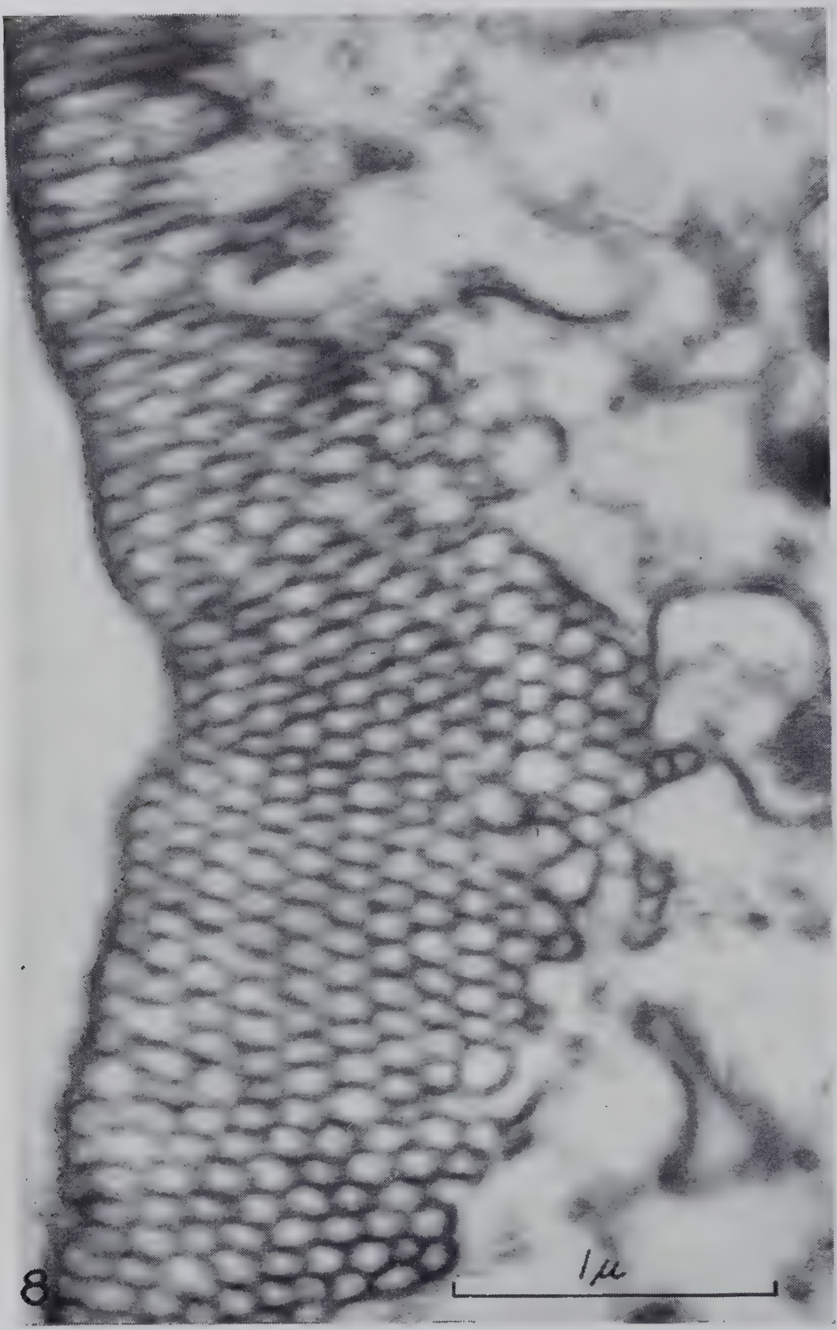

Fig. 8. Electron micrograph of a rhabdomere of a beetle (*Tenebrio molitor*) ommatidium. In this beetle compound eye there are seven retinular cells and one "basal cell" in each ommatidium. The rhabdomeres are on the axial surfaces of the retinular cells. In this micrograph most of the microvilli of the rhabdomere are sectioned obliquely, but a few at the top of the figure have their long axes in the plane of the section.

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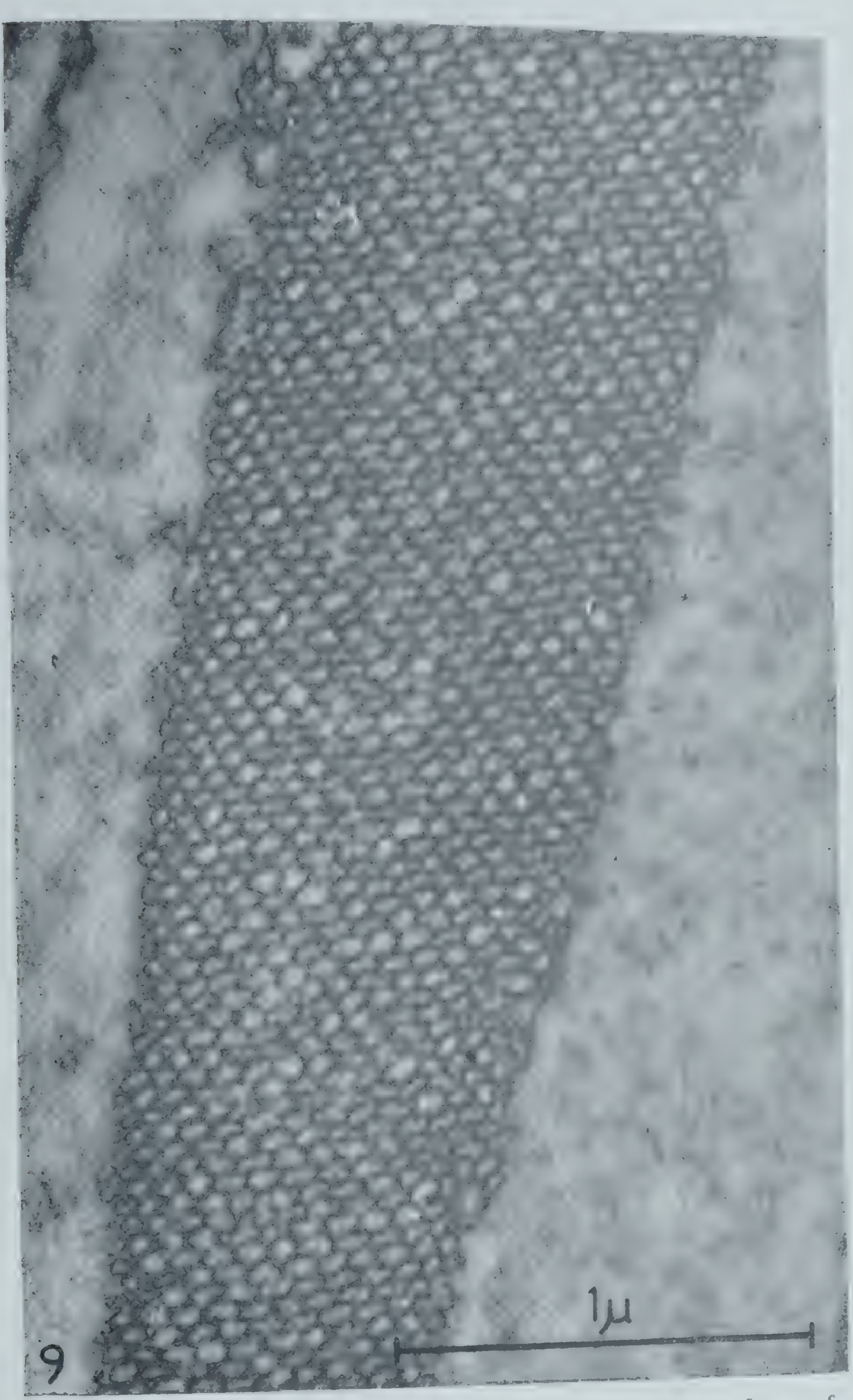

FIG. 9. Electron micrograph of a rhabdomere of the compound eye of a housefly in longitudinal section, showing the polygonal outlines of the rhabdomere's tubular components. From Fernández-Morán (1958).

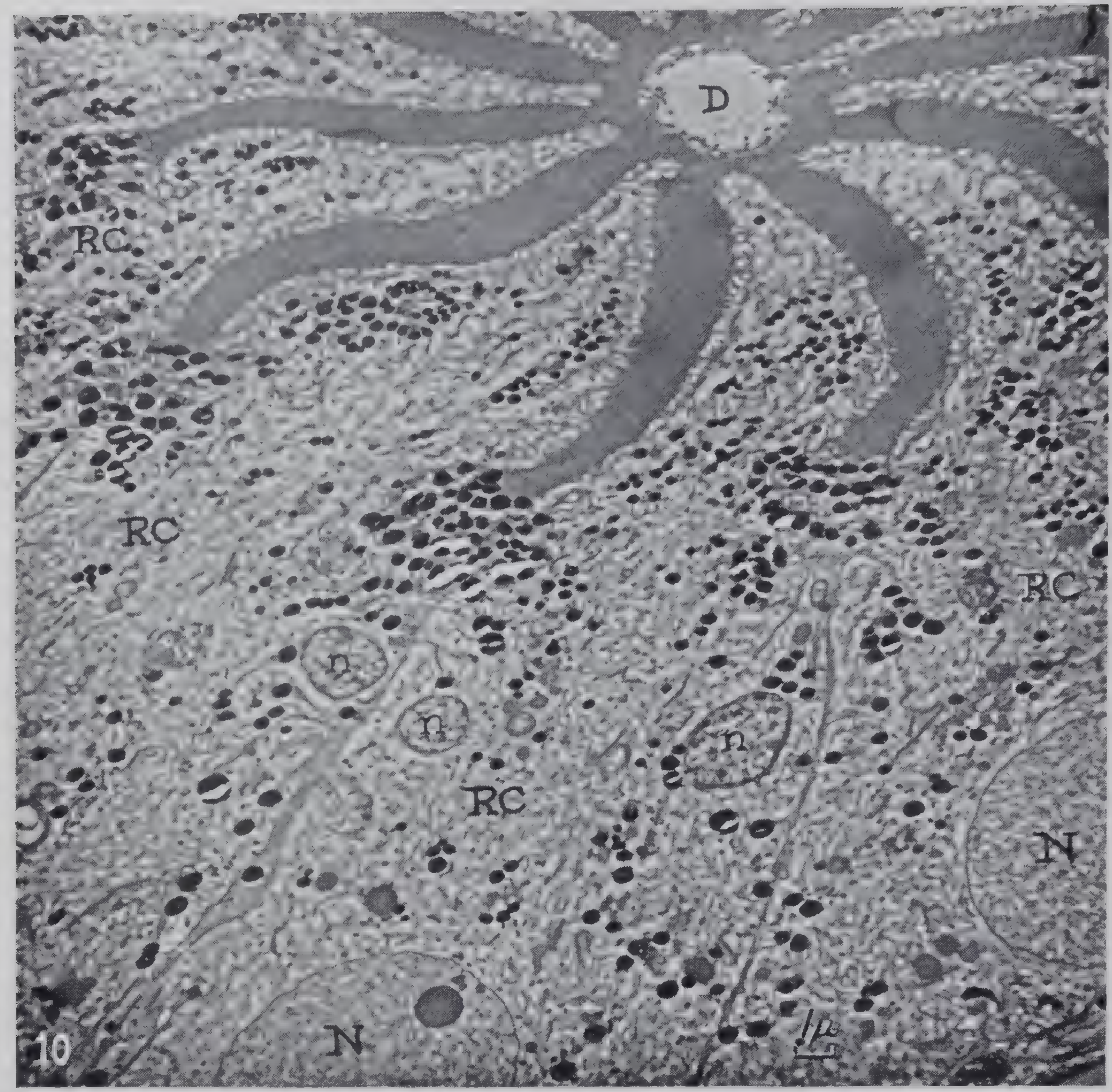

FIG. 10. Low magnification electron micrograph of an ommatidium of the compound eye of *Limulus* in cross section. The rhabdom is composed of the dense spokelike markings as well as the dense ring around the dendritic distal process of the eccentric cell (*D*). The rhabdomeres of indvidual retinular cells (*RC*) are V-shaped; the spokelike markings are composed of parts of two closely applied rhabdomeres. The large nuclei (*N*) belong to the retinular cells, the small nuclei (*n*) belong to small spindle-shaped pigment cells that are sandwiched between the retinular cells.

rhabdomeres of neighboring retinular cells. Figures 11, b and 11, c show the appearance of the microvilli in oblique and cross section. The long axes of these microvilli lie in transverse planes and are normal to the retinular cell's surface so that there results a radial symmetry in the fine structure of the rhabdom. In insect ommatidia the tubular components of the rhabdomeres of each retinular cell have different orientations, and many investigators have speculated that this might form a basis for the ommatidium to act as a polarized light analyzer.

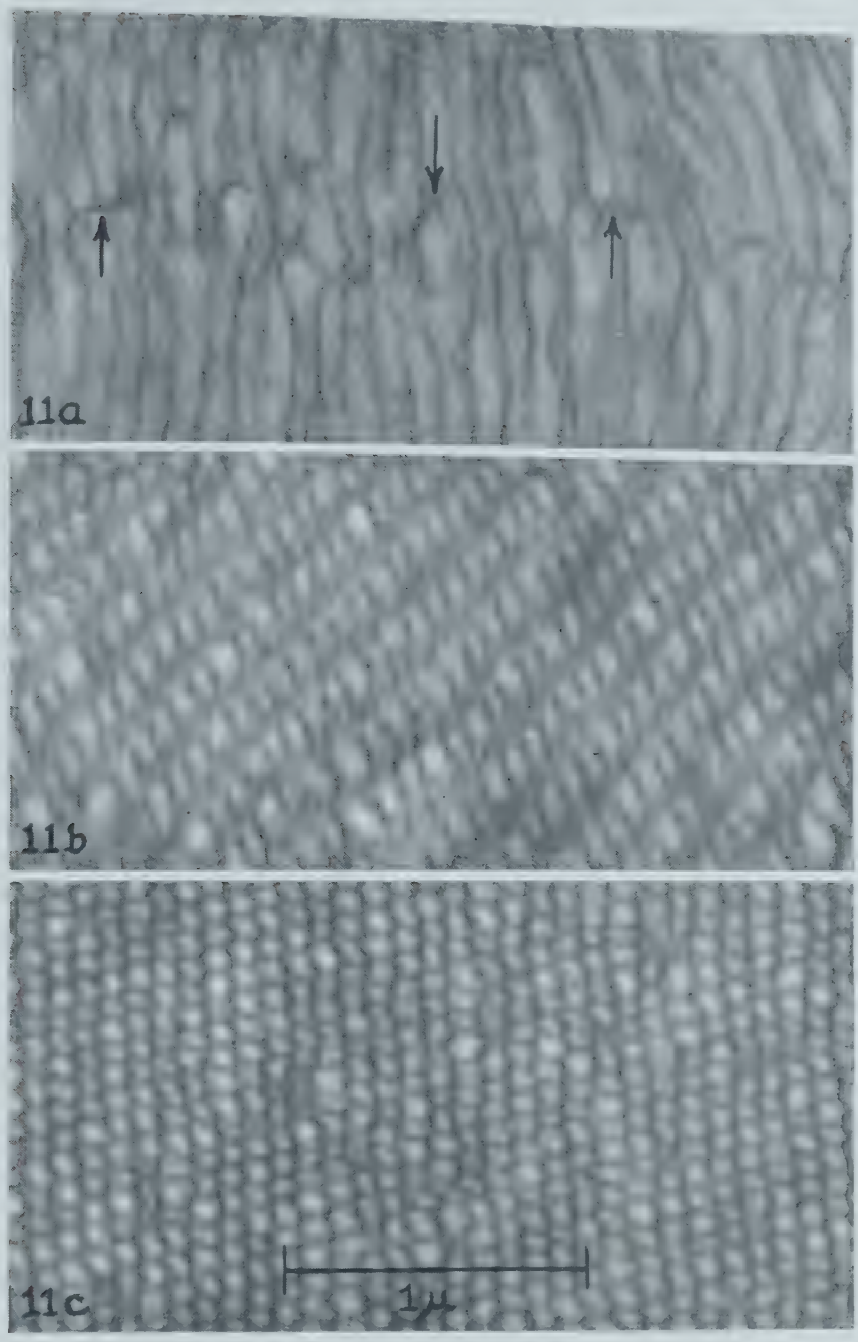

FIG. 11. Electron micrographs at higher magnification of parts of the rhabdom of the compound eye of *Limulus*. The rhabdomeres are tightly packed microvilli of the retinular cells' surfaces. a. Taken from a cross section of the ommatidium. The long axes of the microvilli are in the plane of the section. The arrows mark the plane of abutment of the rhabdomeres of adjacent retinular cells. b and c. Sections which cut the microvilli of rhabdomeres obliquely and at right angles to their long axes, respectively. From Miller (1958a).

The arthropod photoreceptor structures described above were all obtained from adult specimens. William Marks of the Jenkins Department of Biophysics at Johns Hopkins University reports (personal communication) that the rhabdomeres of retinular cells in the ocellus of an insect larva, probably the caterpillar *Isia isabella,* when examined by means of the electron microscope also show the structure of closely packed microvilli that has been reported in adult arthropods.

The arthropod rhabdomere is unlike the vertebrate rod and cone outer segments in that no trace of a ciliary structure has been observed. Indeed, it had been thought that the arthropods are characterized by the absence of cilia. However, that this is not the case has been shown by a recent electron microscopic investigation. Gray and Pumphrey (1958) find that the chordotonal sensilla of an insect is derived from a cilium.

2. *Mollusks*

In mollusks, photoreceptor structures are found in a variety of shapes. The cephalopods usually have visual receptor cells that are rod-shaped. These rods may possess *Stiftchensäume* (little pin borders) and often contain an "axial filament" in the rod's axis (Hesse, 1900). A recent electron micrograph investigation by Wolken (1959) has shown that the *Stiftchensäume* of the rods of *Sepia* and *Octopus* have a fine structure similar to that of the rhabdomeres of arthropods. They consist of tightly packed tubules and resemble a honeycomb.

The visual cells in the retinas of such bivalved mollusks as *Arca* and *Lima* and the pelagic heteropods *Carinaria* and *Pterotrachea* have *Stiftchensäume* in the shape of caps or mantels (Hesse, 1900). The eyes of several pelecypods such as the scallop *Pecten* and *Spondylus* are interesting in that they have two retinas. In the distal retina the visual cells have *Stiftchensäume* that cap the cells, while in the proximal retina the visual cells are rod-shaped and contain axial filaments. Hesse (1900, 1902) observed that the *Stiftchensäume* in the receptor cells of several gastropod and pelecypod eyes seemed to be composed of many very fine hairlike threads. In preparations stained with hemotoxylin he saw at the base of each thread a small darkly stained mass which he likened to the basal body of a cilium. His interpretation that the photoreceptor structure in these eyes is derived from cilia met with strong opposition (Smith, 1906; Küpfer, 1916); nevertheless, a recent electron microscopic investigation has shown that Hesse was correct. The distal sense cells in the eye of *Pecten* have globular appendages that are derived from cilia (Miller, 1958b). The distal sense cell appendage is composed of concentric lamellae, each of which is seen to be continuous with a ciliary stalk and basal body (Fig. 12). Figure 13, a is an electron micrograph

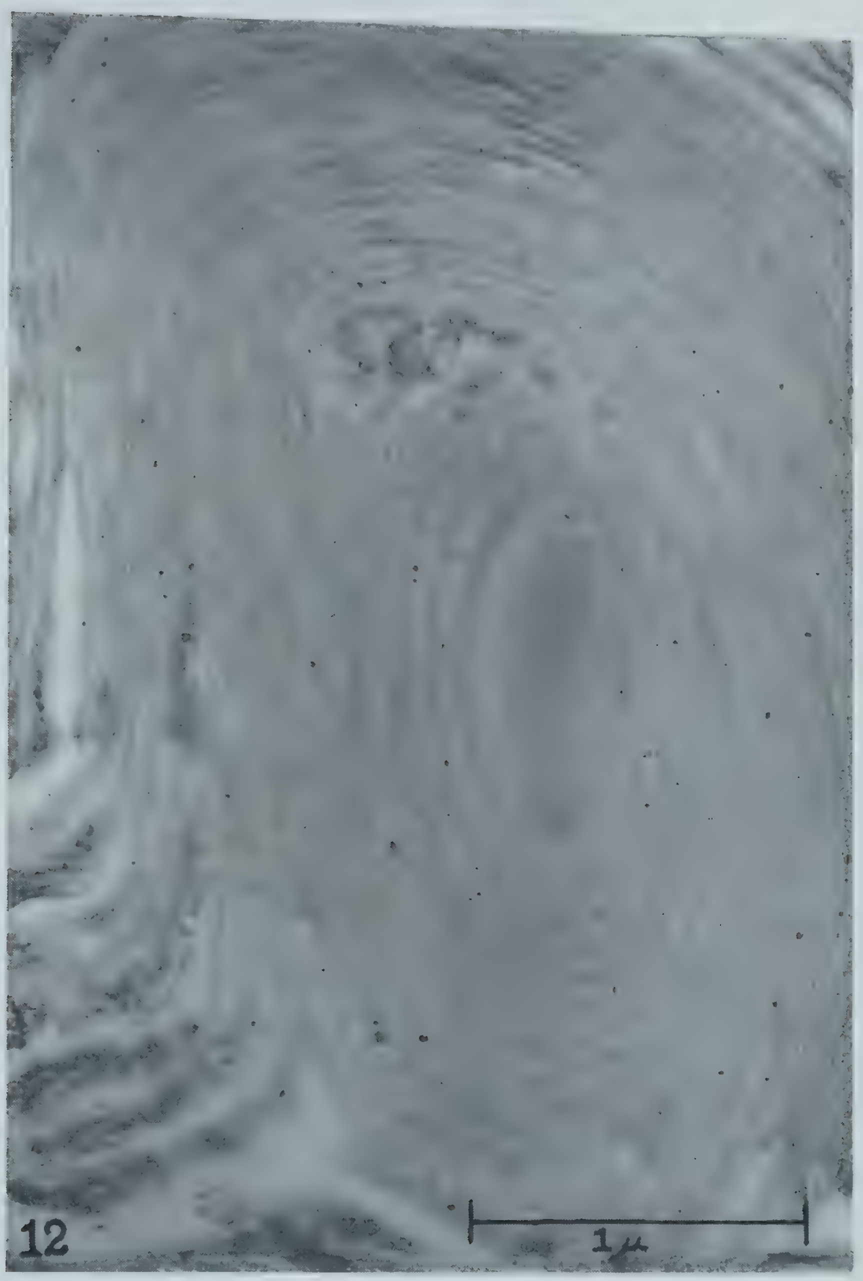

FIG. 12. Electron micrograph of distal sense cell appendage of the eye of the mollusk, *Pecten irradians*. The distal sense cell appendage is composed of concentric lamellae. Each lamella is continuous with the stalk and basal body of a cilium. The ciliary stalks, within which are ciliary filaments, are seen in the lower left of the figure. From Miller (1958a).

of an oblique section through the free ends of parts of three rods (*R*) in the proximal retina of the eye of *Pecten*. Long microvillus extensions are seen to emanate from the surfaces of the rods. Though there is no direct evidence that these microvilli contain visual pigments, on the basis of their appearance they are tentatively assumed to be photoreceptor structures. The axial filament (*Ax.F.*) is seen in the center of one rod and is

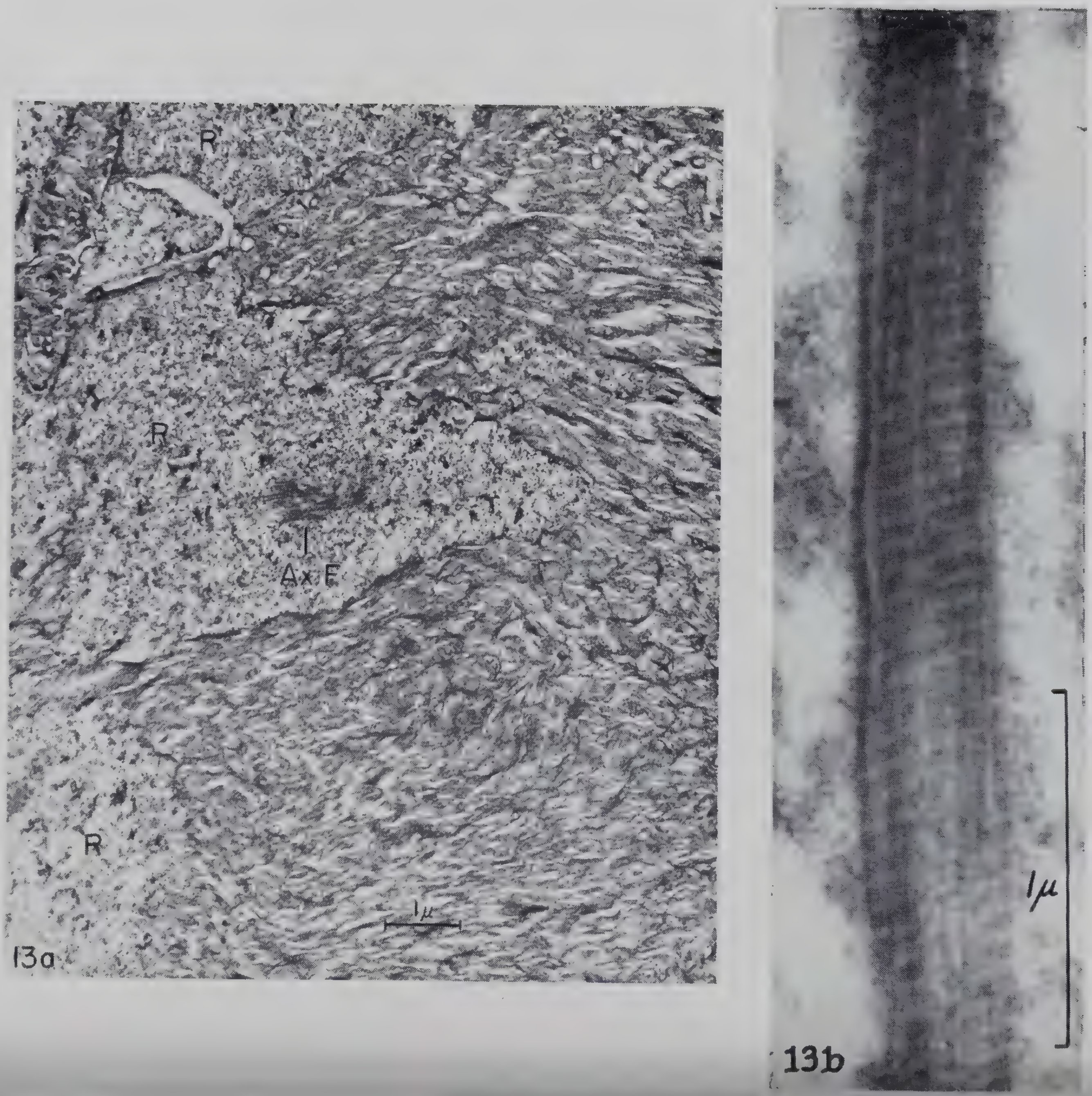

FIG. 13. Electron micrographs of the sense cells of the proximal retina of *Pecten*. a. Micrograph showing the free ends of three rods (*R*). The matted microvilli extending from the surfaces of the rods are assumed to be photoreceptor structures. The obliquely sectioned axial fibril (*Ax.F*) is seen in the center of one rod. b. A higher magnification micrograph of the axial filament in longitudinal section. It is seen to be composed of fibrils with a banded appearance which closely resembles kinetodesmal fibrils.

shown at higher magnification in longitudinal section in the electron micrograph, Fig. 13, b. It may be significant that this filament is not, as was previously thought (Hesse, 1900; Küpfer, 1916), composed of neurofibrils. Rather, because it is intracellular and because of the periodicity of its platelike elements (cf. Sedar and Porter, 1955; Gray and Pumphrey, 1958), the axial fibrils resemble kinetodesmal fibrils, structures that are characteristically associated with the basal bodies of cilia.

3. *Protophytes*

The phytoflagellates, unicellular organisms with characteristics of both the plant and animal kingdoms, contain reddish eyespots, or stigmata, which are probably photoreceptors (Hartshorne, 1953). The eyespots in these organisms are built into chloroplasts, and in addition they are often found in close association with a flagellum. In an electron microscope investigation Sager and Palade (1957) have shown that the eyespot of *Chlamydomonas reinhardi* consists of hexagonally packed granules that are arranged in either two or three layers between the disks of the chloroplast. Figure 14, a, which is reproduced from their paper, is an electron micrograph of a section taken perpendicular to the two layers of granules (e_1 and e_2), which are located just beneath the cell membrane (*cm*) but within the chloroplast envelope (*ce*) and between chloroplast disks (d_1 and d_2). Figure 14, b is a micrograph of a section taken obliquely through a layer of granules, showing close hexagonal packing about the granule marked with a cross. The granules are between 100 and 140 mμ in diameter, have a dense core (*cr*), and are not bounded by membranes.

The eyespot of *Euglena* is a cluster of large granules located near (though not connected with) that organism's flagellum (Wolken and Palade, 1952; Wolken, 1956). In the case of another organism, *Chromulina*, the granules of the eyespot are membrane limited. Investigation of *Chromulina* by the electron microscope has also shown that its eyespot is juxtaposed to a small "internal flagellum" which is not visible by the light microscope (Rouiller and Fauré-Fremiet, 1958).

The spermatozoid of a plant, the brown alga *Fucus*, also shows coaptation of a flagellum and eyespot (Manton and Clarke, 1956). The hind flagellum is at first free, then closely applied to the cell membrane over the area of the eyespot and free thereafter.

4. *Amphioxus*

This invertebrate chordate combines prominent features of invertebrates (e.g., flame cell excretory organs) and vertebrates (e.g., hollow dorsal nerve cord) alike, and it is for this reason that the morphology of its photoreceptors is of particular interest. While amphioxus has no

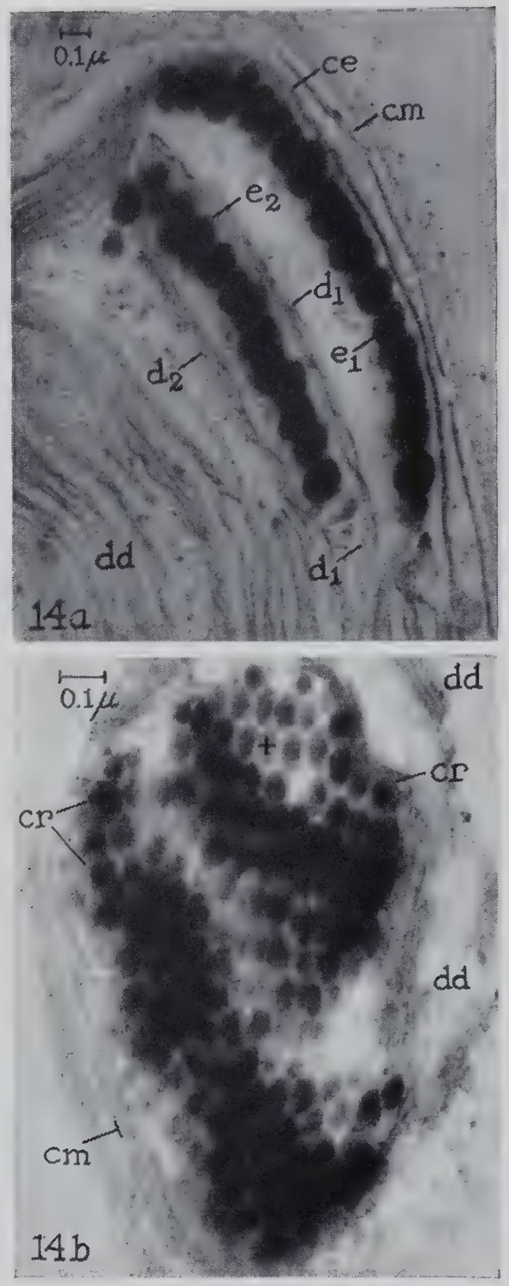

FIG. 14. Electron micrographs of the eyespot of *Chlamydomonas*. a. Normal to the eyespot which consists of rows of granules (e_1) and (e_2). The row (e_1) is between the chloroplast envelope (*ce*) and chloroplast disk (d_1). Row (e_2) is between disks (d_1) and (d_2). Other chloroplast disks are seen (*dd*). The cell membrane is marked (*cm*). b. In the plane of one of the rows of granules. The granules have a dense core (*cr*). Hexagonal packing of the granules is seen around the granules marked with a cross. From Sager and Palade (1957).

eyes, there are located along the posterior nerve cord clusters of cells with *Stiftchensäume*, which Hesse (1898) regarded as photoreceptors. Peter Satir of the Rockefeller Institute has investigated these cells with the electron microscope and reports that the *Stiftchensäume* resemble arthropod rhabomeres; they are composed of microvilli of the Hesse cell's border and have a honeycomblike structure (personal communication). Satir has also found previously undescribed cells located near the infundibular organ which are similar to both the vertebrate visual cells and the distal sense cells in the retina of *Pecten* in that they have appendages with a lamellar structure which are ciliary derivatives. He believes these cells, as well as those of Hesse, to be photoreceptors, though it would, of course, be desirable to have more than morphological evidence as to their function.

III. VISUAL PIGMENTS

A. Rhodopsin

The importance of photoreceptor structures as specialized organelles of visual cells lies in the fact that they contain visual pigments; for it is the visual pigments that by their absorption and other physical and chemical properties determine the limits of spectral range and sensitivity of eyes. For vertebrate eyes, the presence of pigments in rod outer segments was noticed by a number of early observers. Thus H. Müller (1856) (quoted in Kühne, 1878) wrote of the frog retina: "The substance of rods is often seen to be reddish . . . if it is of sufficient thickness; as for instance when a rod is viewed on end, or several lying together, one over another." However, the history of modern knowledge of visual pigments may be said to begin in 1877 with the observation of F. Boll that the reddish color of vertebrate rod outer segments is changed by light. Boll's discovery stimulated W. Kühne (1878) to study the nature of this reaction in detail. The reddish rod pigment Kühne called visual purple, or rhodopsin. When rhodopsin, either in intact rods or in bile-salt solution, is exposed to light, the color becomes briefly chamois, orange, then yellow, and finally colorless. The yellow product, which is relatively stable, Kühne called visual yellow or xanthopsin. The final colorless product, the presence of which was detected by the appearance of a greenish flourescence to ultraviolet radiation (Kühne and Ewald, 1878), he called visual white or leucopsin. Kühne found further that rhodopsin regenerates slowly in the dark from visual white not only in the intact retina (provided it is left in contact with the pigmented epithelium and choroid), but also in bile-salt solution.

Rhodopsin is a conjugated protein (Wald, 1935) with a molecular weight of about 40,000 (Hubbard, 1954). It is a combination of a species-specific protein called opsin, and a light-absorbing carotenoid chromophore, retinene, which may be split from the protein either by the action of chemicals that destroy rhodopsin, such as chloroform, or by the action of light (Wald, 1935). Retinene, which is vitamin A aldehyde, $C_{19}H_{27}CHO$ (Ball *et al.*, 1948a), exists in a number of geometrical configurations, the *cis-trans* isomers (which are illustrated in Fig. 15, from Hubbard and Kropf, 1958). Retinene is photosensitive, the action of light being to isomerize the molecule. Stereoisomerization occurs as a result of this action of light whether the retinene is alone in solution or whether it is attached to opsin (Hubbard and Wald, 1952; Hubbard and Kropf, 1958). Rhodopsin is the combination of one particular isomer, neo-*b* (11-*cis*) retinene, tightly bound to opsin. According to Hubbard (1958), the action of light on rhodopsin is to change the configuration of the neo-*b* retinene to the all-*trans* form. This stereoisomerization results in the retinene changing configuration and hence partially breaking away from the opsin, causing the formation of an orange photoproduct called lumi-rhodopsin by Wald and his colleagues (Lythgoe and Quilliam, 1938; Wald *et al.*, 1950). That the transformation of rhodopsin to all-*trans* lumi-rhodopsin is actually a photochemical reaction has been shown by Hubbard (1958), who found that the action of heat on rhodopsin produces 11-*cis* retinene and denatured protein, whereas light invariably gives all-*trans* lumi-rhodopsin. Lumi-rhodopsin, then, is all-*trans* retinene loosely bound to opsin. At temperatures above –20° C. in the dark, all-*trans* lumi-rhodopsin changes to a new orange compound called all-*trans* meta-rhodopsin (Wald *et al.*, 1950), a change that does not involve further stereoisomerization of the chromophore (Hubbard and Kropf, 1958). If the lumi- and meta-rhodopsin are now further exposed to light, a further photochemical

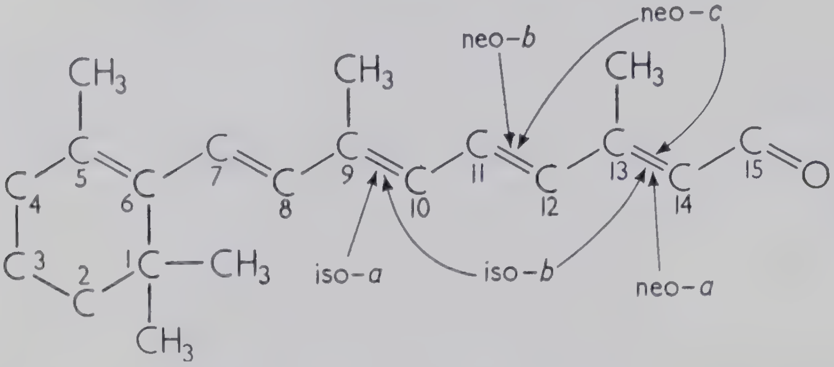

FIG. 15. Structural formula of retinene, which is shown in the all-*trans* configuration. The arrows indicate the double bonds which are in *cis* configuration in the various *cis* isomers. The neo-*b* isomer (11-*cis*) is the chromophore of rhodopsin. From Hubbard and Kropf (1958).

reaction occurs. The all-*trans* retinene is isomerized producing an equilibrium mixture of all the isomers, one of which is neo-*b*, which, since it is attached to opsin, is regenerated rhodopsin. Another isomer produced by the action of light on lumi- and meta-rhodopsin is iso-*a* retinene, which in combination with opsin produces isorhodopsin, a pigment that is probably not present *in vivo* (Chase, 1937; Collins and Morton, 1950; Hubbard and Wald, 1952). The combinations of the other isomers of retinene with opsin are unstable and in the presence of water above –15° C. hydrolyze first to the relatively stable indicator yellow (deep chrome at acid, pale yellow at alkaline pHs; Chase, 1936; Lythgoe, 1937) in which retinene is still attached to opsin (Collins, 1953). Finally separation occurs, yielding the colorless protein, opsin, and yellowish vitamin A aldehyde, retinene. This scheme of the rhodopsin cycle is shown in Fig. 16 (from Hubbard and Kropf, 1958).

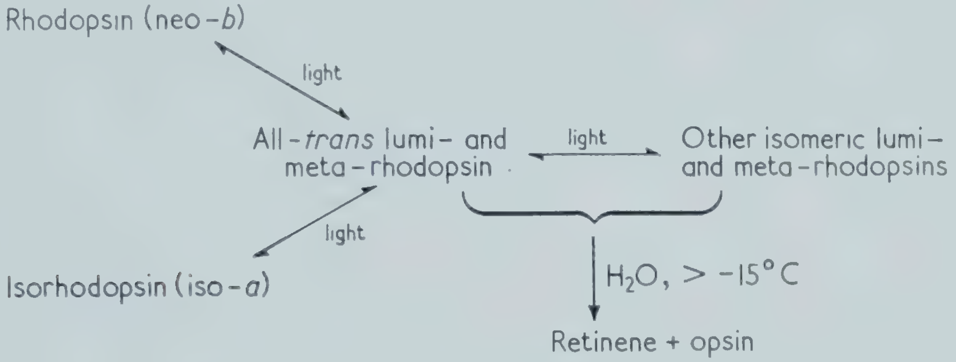

FIG. 16. Hubbard's and Kropf's general scheme of photochemical reactions involving retinene and opsin. From Hubbard and Kropf (1958).

The visual process is ultimately dependent for its rhodopsin on body stores of vitamin A (Tansley, 1933; Dowling and Wald, 1958). Retinene, entirely free from opsin, is in equilibrium in the retina with vitamin A (Wald, 1935), which exists in the same geometrical *cis-trans* isomers as retinene. The interconversion of vitamin A and retinene *in vitro* is catalyzed by alcoholdehydrogenase and coenzyme I (Bliss, 1948, 1951; Wald and Hubbard, 1948–1949). The detached retinene may be isomerized by light or by an isomerase *in vitro* (Hubbard, 1956). It has been argued that the photoisomerization of retinene does not play a role in the *in vivo* regeneration of rhodopsin (Rushton, 1957).

The nature of the bond or bonds between retinene and opsin is unknown. Analytical studies of reactions of retinene with amino acids point to a carbon to nitrogen bond (Ball *et al.*, 1948b, 1949; Collins, 1953). Evidence for this is based on the fact that if solutions of retinene are mixed with certain proteins or amino acids, there results a yellow product that spectroscopically resembles indicator yellow and is an acid base indicator. Other experiments suggest that retinene condenses with sulfhydryl groups of opsin (Wald and Brown, 1952).

B. Other Vertebrate Visual Pigments

The earliest surveys of visual pigments of vertebrates revealed the presence of more than one rod pigment. Thus Köttgen and Abelsdorff (1896) confirmed even earlier observations that the pigments of the rods of some fresh-water fish are not reddish like those of land vertebrates, but rather purple colored. Wald, who has particularly interested himself in this problem, has confirmed and extended these observations. He finds that the rods in the retinas of most marine fish, as well as land vertebrates, contain rhodopsin. But the rods in the retinas of most fresh-water vertebrates contain the purple visual pigment which he calls porphyropsin. Furthermore, the retinas of animals that spend their lives in a mixture of environments, either fresh water, salt water, or land, contain mixtures of rhodopsin and porphyropsin (Wald, 1939, 1941, 1945–1946, 1946, 1955, 1957). The difference between rhodopsin and porphyropsin is in the form of vitamin A that is the precursor of the chromophore. The chromophore of rhodopsin is the aldehyde of vitamin A_1, $C_{19}H_{27}CH_2OH$ (retinene$_1$). Vitamin A also exists in a dehydro form, vitamin A_2, $C_{19}H_{25}CH_2OH$ (Farrar *et al.*, 1952), and it is the aldehyde of vitamin A_2 (retinene$_2$) which is the chromophore of porphyropsin. Wald believes that the protein moieties of all rod pigments are sufficiently similar to be designated "scotopsins." Using a method of partial bleaching by light of different wavelengths, Dartnall has found evidence for the existence of vertebrate visual pigments with absorption spectra that differ from rhodopsin and porphyropsin (summarized in Dartnall, 1957, pp. 158–180; see also Crescitelli, 1958). Dartnall suggests that the different absorption properties of these pigments could possibly be due to species differences in the protein opsins.

Unlike outer segments of the rods, the outer segments of the cones do not have sufficient amounts of pigment in order to be seen with the naked eye. There is, however, mounting evidence that cone outer segments do contain visual pigments. Wald (1937) isolated a red-absorbing pigment from the chicken retina, presumably from the outer segments of the cones, which he called iodopsin. It is a combination of 11-*cis* retinene$_1$, the same isomer found in rhodopsin, and the protein moiety thought to be characteristic of cones and named "photopsin" by Wald (Bliss, 1946; Wald *et al.*, 1955). In addition, the combination of photopsin with retinene$_2$ has been synthesized *in vitro* (Wald *et al.*, 1953) yielding another red-sensitive pigment, "cyanopsin," which could presumably be present in the cones of fresh-water fish. Rushton (1958, see Section IV) has demonstrated the existence of a green-sensitive and a red-sensitive cone pigment in the living human fovea. Hanaoka and Fujimoto (1957), by means of a microspectrophotometer, have detected photosensitive pigments in single cone outer segments of a fish, the carp.

C. Invertebrate Visual Pigments

The existence of visual receptor cells with reddish-colored organelles in the eyes of many invertebrates has been known for over a century (Krohn, 1842; Hensen, 1865; Schultze, 1868: all cited in Kühne, 1878). Kühne (1878) was reluctant to consider the colored substances of invertebrate rods as visual pigments because their color did not appear to be altered by the action of light. Recent investigations of the squid eye (St. George and Wald, 1949; Hubbard and St. George, 1958) and the compound eye of the lobster (Wald and Hubbard, 1957) have shown that the red pigments of the rhabdoms of these retinas are indeed visual pigments. In both cases the chromophore of the visual pigment is 11-*cis* retinene, and the action of light is to isomerize the retinene to the all-*trans* form. The difference, however, between these invertebrate pigments and those of vertebrates is that whereas red vertebrate meta-rhodopsin is unstable above –15° C. and hydrolyzes to yellow retinene and protein, the meta-rhodopsins of these two invertebrate retinas are stable even at room temperature. Since the absorption spectra of rhodopsin and meta-rhodopsin are similar, the color of these invertebrate photoreceptors does not seem to be affected by light; thus there is no visible bleaching, but nevertheless a photochemical reaction.

By treating the compound eyes of the blue crab, *Callinectes*, and *Limulus* with either 10% ethyl alcohol or 10% Formalin, Bliss (1942–1943) was able to extract light-sensitive pigments that with photolysis liberate retinene. Visual pigments have been extracted from the eyes of three other arthropods: euphausiid crustaceans (Kampa, 1955), honeybees (Goldsmith, 1958), and houseflies (Bowness and Wolken, 1959). The honeybee visual pigment, the chromophore of which is retinene$_1$, and that of the housefly, which is of unknown chemical composition, are unique in being water soluble; the vertebrate visual pigments can, apparently, only be brought into solution by means of detergents such as bile salts (Kühne, 1878) or digitonin (Tansley, 1931).

D. Location of the Visual Pigments

Rhodopsin is easily visible in rod outer segments. It comprises 3.7% of the wet weight of cattle rod outer segments and 10% of the wet weight of those of the frog (Hubbard, 1954). Its exact distribution and location in the lamellated rod outer segments is, however, unknown. Although a number of molecular models have been postulated (e.g., Wolken, 1956), neither polarization nor electron microscopic data give evidence that would allow for a unique interpretation. The same applies to the cone outer segments and invertebrate photoreceptors. However, a recent modification of a visual pigment staining technique developed by Stern (1905) gives promise of achieving this goal. Stern discovered that dark-adapted frog rod outer segments, when fixed in 2.5% platinum chloride, were stained an intense orange, whereas light-adapted outer segments similarly fixed were colorless. The same results were obtained using the retinas of warm-blooded animals, the rabbit and cat. Recently, Fernández-Morán (1959, and personal communication) reports preliminary studies by which he has succeeded in adapting this method for use with the electron microscope. Vertebrate retinas are, by Fernández-Morán's technique, rapidly frozen in liquid helium and then at still very low temperatures the water in these retinas is substituted by alcohol in which platinum chloride is dissolved. The retinas are then embedded in methacrylate at a low temperature. Fernández-Morán (1959) finds that electron micrographs of light-adapted outer segments show "an intermediate dense line 15 to 20 A. thick, which had not been detected previously between the double layers [of the disklike units]." In preliminary experiments with dark-adapted outer segments Fernández-Morán reports "an electron dense component is found, associated with the double layers . . ." It will be interesting to see if this research leads to the exact localization of visual pigments within photoreceptor structures.

E. Summary

Visual pigments in the eyes of every animal that has been investigated consist of a protein molecule to which is bound a carotenoid chromophore. Furthermore, thus far in every case examined, without exception, the chromophore is one particular isomer of a form of vitamin A aldehyde, the 11-*cis* form. Vitamin A aldehyde is photosensitive, and the action of light on visual pigments is to cleave partially the photosensitive chromophore from the protein by isomerizing vitamin A aldehyde from the 11-*cis* form to the all-*trans* form. A point of major interest is whether future investigations will uncover exceptions to this pattern.

We have focused our attention on receptor structures and pigments, but visual receptors are, of course, very complex cells that perform a multitude of functions (including, perhaps, filtering and focusing of light, photomechanical movements, and synaptic functions). A most important function of visual receptor cells is the production of nervous excitation. However, the events that occur between the photochemical reaction and nervous excitation are entirely unknown.

IV. Photoreceptor Physiology

A. Visual Pigments and Vision

It is clear from recent physiological research that the vertebrate eye can detect very small amounts of light. Hecht *et al.* (1942) found that the dark-adapted human eye can see about 100 quanta incident at the cornea. When this figure was adjusted for the estimated fraction of light absorbed by rhodopsin after losses both at the cornea by reflection and in the ocular media by absorption, they calculated that something on the order of five to fourteen absorbed quanta can be seen. In these experiments an area of the retina containing about 500 rods was stimulated, and it is improbable that more than one quantum would be absorbed by any one rod. Therefore, Hecht and associates concluded that the absorption of one quantum by each one of five to fourteen rods may result in a visual sensation. Similar results were obtained using a statistical method of analysis by Hecht *et al.* as well as independently by Bouman and Van Der Velden (1947). Although there is some controversy about the exact figure for the absolute threshold of vision, the significant finding is that very few absorbed quanta can be seen (reviewed by Pirenne, 1956).

Although one absorbed quantum is as effective a stimulus as any other, the eye is not equally sensitive to incident light of all wavelengths. The dark-adapted human eye is most sensitive to blue-green light with a wavelength of about 500 mμ, the wavelength at which human visual purple appears most dense. The well-documented quantitative correspondence between the absorption properties of human visual purple and the visibility curves of persons under conditions of dim illumination is convincing proof of the function of visual purple as a visual pigment. As early as 1894 König found good agreement between the absorption spectrum of human rhodopsin and the visibility curves both of persons with normal vision stimulated at threshold intensities and totally color-blind persons. Hecht and Williams (1922) carefully compared human scotopic visibility data with the absorption spectra of monkey and rabbit visual purple in bile-salt solution and found the visibility curve shifted slightly toward the red. Noting that substances dissolved in media of greater refracting power show a shift in their absorption spectra toward the red (Kundt's rule), Hecht and Williams attributed the discrepancy to the fact that the rhodopsin in the living retina is contained in the rod outer segments which have a higher index of refraction than bile-salt solution. Dartnall and Goodeve (1937) discounted the importance of Kundt's rule. Realizing that the absorption of light by rhodopsin should be represented on an equal quantum, rather than an equal energy, basis, they replotted Hecht's and Williams' data and demonstrated better agree-

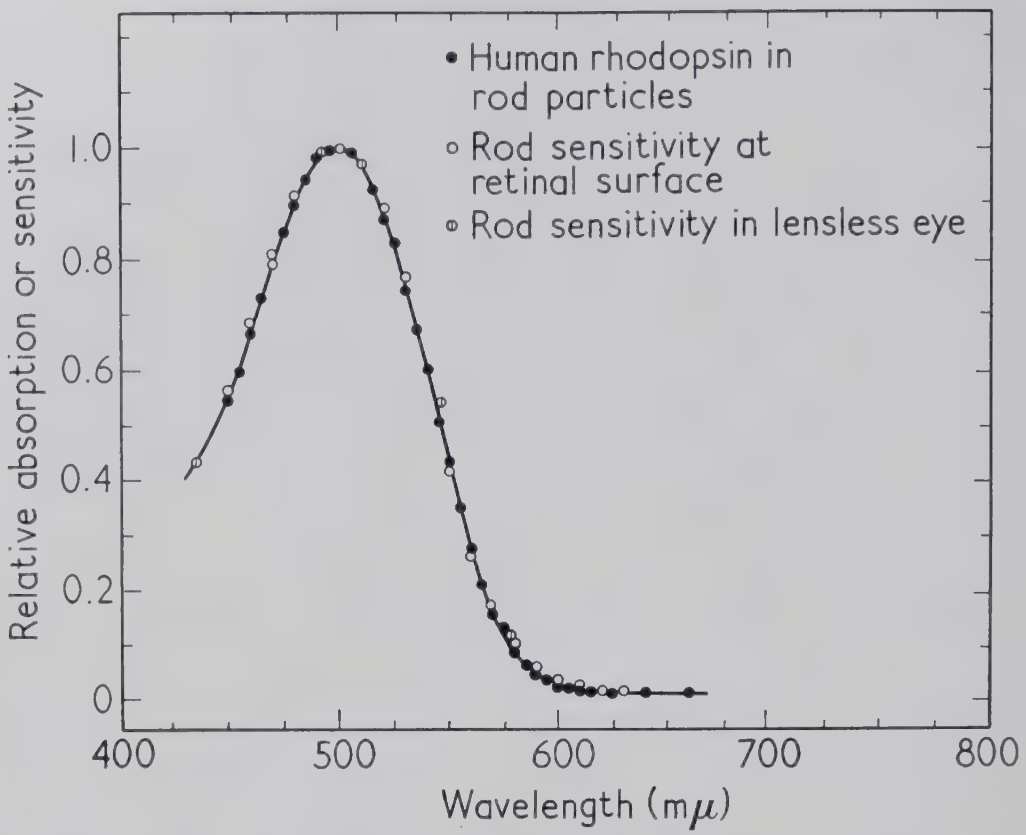

FIG. 17. Scotopic visibility curve for the lensless human eye compared with the scotopic visibility curve of the normal eye corrected for ocular transmission and the absorption spectrum of human rod outer segment particles. From Wald and Brown (1958).

ment between the maxima of the absorption spectrum and visibility curve. Further refinements have recently been made by Crescitelli and Dartnall (1953), who found close agreement between the density spectrum of human rhodopsin and Crawford's (1949) human scotopic visibility data, and Wald and Brown (1958), who compared scotopic visibility data with both rhodopsin in solution and a suspension of fresh human rod outer segments (Fig. 17). Thus rhodopsin is shown to be a visual pigment by the close correspondence between the properties of scotopia on the one hand and the absorption spectrum (and also the photosensitivity spectrum as shown by Schneider *et al.*, 1939) of this pigment present in rod outer segments on the other.

The absorption and sensitivity data obtained using *in vitro* preparations have recently been made more meaningful by an elegant technique whereby the bleaching of rhodopsin may be observed in the living eye (Rushton, 1955; Campbell and Rushton, 1955). Acting along lines suggested by experiments of Abelsdorff (1898) and Brindley and Willmer (1952), Rushton designed a retinal densitometer which can measure small changes in the density of visual pigments by an ophthalmoscopic method utilizing light reflected from the fundus. The eye is presented with a light that flickers orange and green, and the light reflected by the choroid is measured by a photocell. Since rhodopsin is transparent to orange light but absorbs green light, if a match is made between the reflected orange and green light, then a change in the intensity of the reflected green light may be taken to represent a change in the density of rhodopsin. By this method it was found that rhodopsin is absent from the human fovea and optic disk and that its distribution exactly corresponds with that of the rods. The difference spectrum of rhodopsin *in vivo* resembles that of rhodopsin in solution but is significantly displaced toward the red, possibly owing to greater stability of orange photoproducts *in vivo* (Rushton, 1956).

Because they are present in minute quantities, cone visual pigments have proved particularly difficult to extract and characterize. However, Rushton (1955, 1958), by means of his densitometer, has identified two visual pigments, one green absorbing, the other red absorbing, in the living human fovea. Since the fovea centralis is a rod-free area, these must be cone visual pigments. Although color vision theory will not be discussed here (the interested reader is referred to Wright, 1947, and LeGrand, 1957), it can be said that most authorities agree that color sensations are based on the presence of pigments with different absorption properties located in the cone outer segments, and Rushton's findings are evidence in favor of this view.

Complete understanding of the visual process requires knowledge of the quantitative allocation of data of visual phenomena to specific physical constituents of the visual system. Such an understanding was the goal of Selig Hecht, who postulated his theory of vision on the premise that the sensitivity of the eye is related in a simple manner to the concentration of visual pigment in the photoreceptor. To produce excitation at threshold, he reasoned, a constant amount of pigment breakdown products must be produced in a given small amount of time. As the pigment is altered by light, its concentration decreases; there are fewer pigment molecules to absorb the photons and sensitivity is therefore reduced. The concentration of visual pigment, and hence the sensitivity of the eye at a particular time, is determined by the resultant of bleaching and regenerative processes. Since bleaching and regeneration are chemical reactions, the velocity of these reactions must (according to the law of mass action) be directly proportional to the concentration of each reactant raised to an exponent equal to the number of molecules of the reactant as it appears in the equation of the reaction. Hecht found that the theoretical curves based on the threshold assumption and the law of mass action fit remarkably well data of intensity discrimination, flicker, and visual acuity experiments (summarized in Hecht, 1937). Hecht was, of course, aware that the actual visual events are more involved. Today we know that rhodopsin regeneration is not only a dark reaction, but also results from the action of light on all-*trans* meta-rhodopsin. The kinetics of the photochemical reaction appear to be complex and are at present only partly understood (Hubbard and Kropf, 1958; Rushton, 1958). Hecht's simplified scheme meets with difficulties when applied to the data of dark adaptation. Actual correlation of the amount of visual purple with the state of adaptation of animal eyes suggests that sensitivity and concentration of visual purple are not related in a simple way (Tansley, 1931; Granit *et al.*, 1938, 1939). *In vivo* correlations have confirmed this. Thus Rushton and Cohen (1954) report that in man "there is no detectable change in the level of visual purple in the hundred-fold increase in sensitivity which follows the extinction of an ordinary room light." Still, the premise that sensitivity and concentration are related is not invalid. The *in vitro* synthesis of chicken iodopsin and rhodopsin from retinene and opsin roughly parallels human dark adaptation in that cone adaptation and iodopsin synthesis are rapid whereas rod adaptation and rhodopsin synthesis occur more slowly (Wald *et al.*, 1955). The *in vivo* experiments indicate that the rod and cone pigments are bleached by light and their concentration increases during dark adaptation (Campbell and Rushton, 1955; Rushton, 1958). In man, 7 minutes after exposure to intense illumination the rhodopsin is half regenerated and the sensitivity has increased only a small fraction of the total sensitivity increase; but in man, as in other animals examined, the time for return of full sensitivity is the same as the time for the full regeneration of visual purple in the dark (Rushton *et al.*, 1955).

B. Receptor Properties

Observations of the absorption properties of living photoreceptor structures provide just one means of investigating receptor function. Any physical measurement which can be correlated with the state of excitation of the receptor is a promising tool in this study. Thus, for the physiology of the nervous system the recording of the electrical events accompanying nerve cell activity and axon impulse conduction have proved valuable for understanding nervous processes. This same approach would seem natural for elucidating receptor cell processes. Indeed there is a vast literature (reviewed by Granit, 1947) dealing with recordings of the mass electrical response of eyes (electroretinogram, ERG). Because most eyes are composed of millions of receptor cells as well as numerous nerve cells which are not receptors and which are known to be electrically active, the results of such investigations are difficult to interpret. However, the ERG does give an objective measure of the activity of visual organs which can be correlated on the one hand with known receptor properties, and on the other with the visually determined behavior of the organism.

Modern physiological techniques have been developed that make possible the recording of the electrical activity of single cells. Such recordings are accomplished by placing a small number of nerve fibers or a single nerve fiber on an appropriate electrode, or by the placement of a small electrode nearby or within the cell. By the use of these techniques it is possible to record the activity of vertebrate retinal ganglion cells and their axons, the optic nerve fibers (see Hartline, 1938b; Kuffler, 1953; Granit, 1955, pp. 113–150; Barlow *et al.*, 1957). By means of microelectrode techniques, recordings of the electrical activity of still other types of vertebrate neural retinal cells have been studied (MacNichol and Svaetichin, 1958; Tomita *et al.*, 1958). The responses of the retinal neurones are complex, and although, as is the case with the ERG, known properties of the receptors can perhaps be deduced by an analysis of these responses, still these are far from being direct recordings of the receptors. In fact, it has not yet proved possible to record directly the activity of single rod and cone cells.

Although most invertebrate eyes are little less complex than those of the vertebrates, in certain instances there occur circumstances that allow for what is at this time the closest approximation available to single visual sense cell recordings. The recordings from the double retina of the eye of the mollusk, *Pecten* (Hartline, 1938a), and of what is probably a photoreceptor in the caudal ganglion of the arthropod crayfish (Kennedy, 1958) are two examples, but in both of these cases incomplete knowledge of the exact morphology of these structures prevents an unequivocal statement that these are direct sense cell responses. A third example is the compound eye of *Limulus*, the morphology of which is better known (see Section II, B, 1). The nerve impulse discharge of single cells may be recorded when the small bundle of nerve fibers that emanates from an ommatidium is dissected out and its free end placed on one of a pair of electrodes (Hartline and Graham, 1932). Even here, though, it seems probable that this activity is not directly that of the receptors, the retinular cells, but rather the activity is of an associated neuron, the eccentric cell (Hartline *et al.*, 1952). However, it appears that the ommatidium acts as a functional unit. Recordings from ommatidia by means of microelectrodes show that there is a one to one correspondence between spike action potentials recorded within the ommatidium (probably from the eccentric cell) and the discharge of nerve impulses recorded in the ommatidium's bundle of nerve fibers (Hartline *et al.*, 1952; Tomita, 1956, 1957). When recorded within the ommatidium, the spike action potentials are positive going and are superimposed on a more slowly changing positive potential which is thought, but not proved, to be associated with the activity of the receptors. The magnitude of this potential is directly proportional to the frequency of the spike action potentials (MacNichol, 1956; Fuortes, 1958).

The properties of this relatively simple sensory unit, then, may be investigated by analyzing its nerve fiber response. When light is directed onto an ommatidium, the discharge of spike action potentials commences after a short latency, which is shorter the higher the intensity of the stimulus. This is illustrated in Fig. 18 (from Hartline, 1940) in which are four records of the discharge of nerve impulses from an ommatidium. The intensity of the light stimulus was brightest for the upper record and was decreased by a factor of 10 for each succeeding lower record. The duration of the stimulus is indicated by the dark line under the spikes. The discharge is seen to be rapid at first, gradually tapering to a steady frequency which was approximately linear with the logarithm of the stimulus intensity (Hartline and Graham, 1932). The tapering of the discharge has its counterpart in a familiar attribute of sensation: adaptation. The intensity versus frequency relationship is likewise found to be applicable to other senses as well as vision and is described as the Weber-Fechner "law."

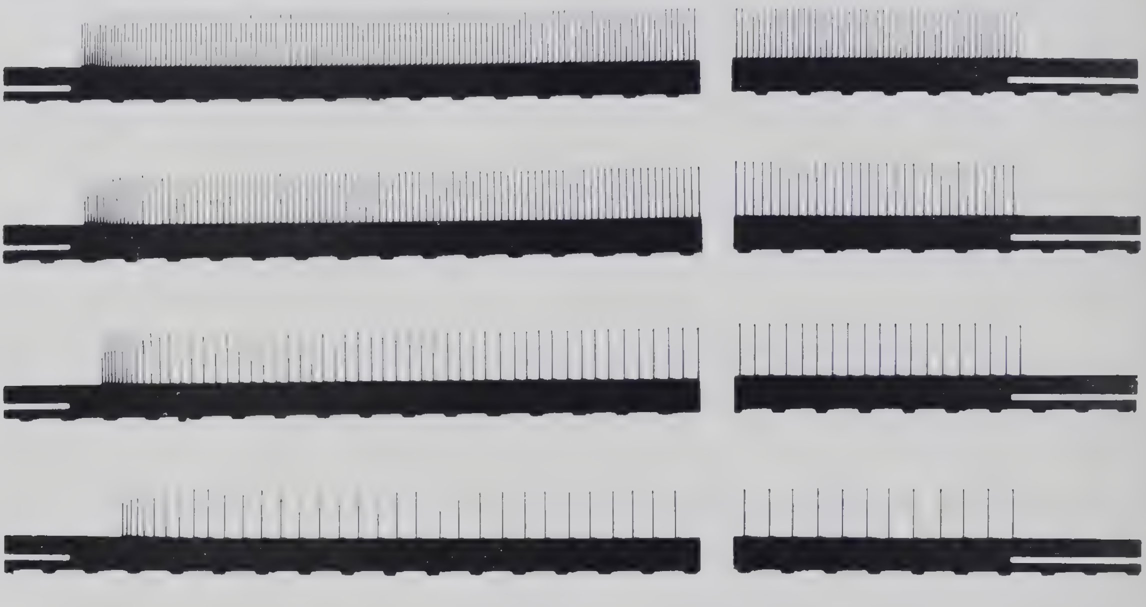

FIG. 18. Four records of spike discharge in single optic nerve fiber of *Limulus*. The intensity of the stimulating light was highest in the upper record and lower by a factor of 10 in each succeeding lower record. The darkening of the space under the spikes indicates the duration of stimulation. One-fifth of a second between time marks. From Hartline (1940).

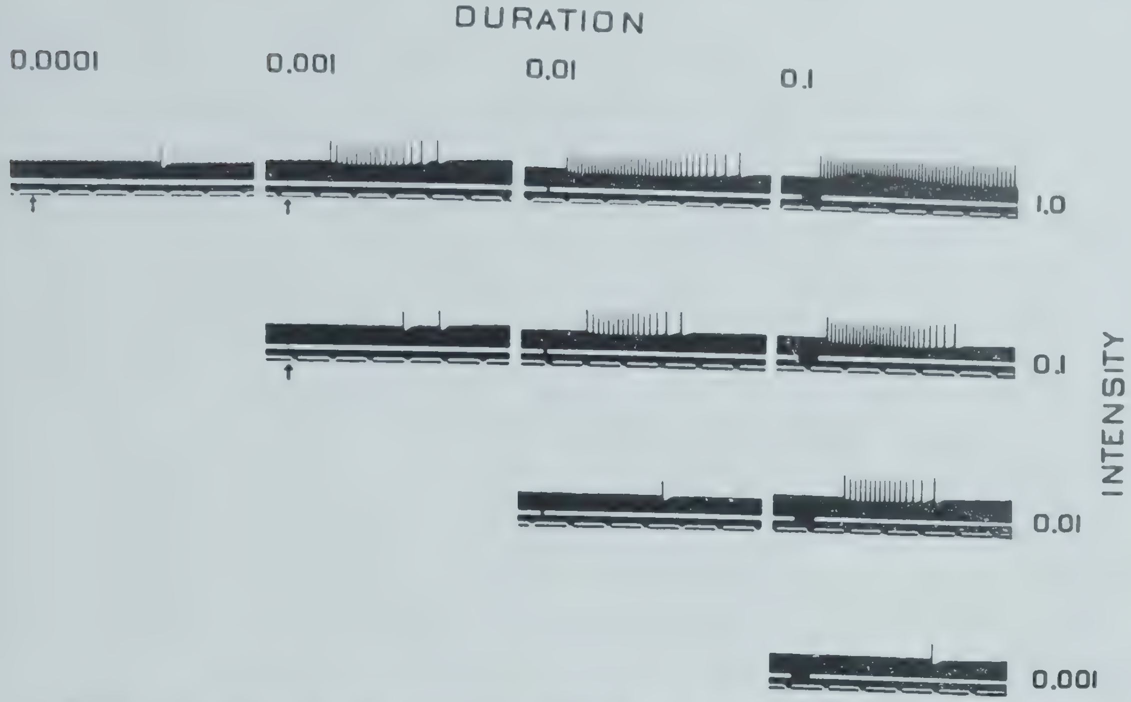

FIG. 19. Records of spike action potentials in single *Limulus* optic nerve fibers in response to light flashes of various intensities and durations. Responses in horizontal rows are to flashes of constant intensity and varying duration, those in vertical rows are to flashes of constant duration and varying intensity. The responses in diagonal rows are approximately equal. This demonstrates that for short flashes the energy content of the flash, regardless of its time distribution, determines the response. From Hartline (1934).

By carefully controlling the stimulus and analyzing the response of the ommatidium, it is possible to compare the receptor unit's properties on the one hand with those of the visual pigments and on the other with the results of psychophysical visual studies (Hartline, 1942). For example, the effect of a photochemical reaction, according to the law of Bunsen and Roscoe, is a function of the energy content of the light flash regardless of its time distribution, and it is found that, for both human vision and *Limulus* single optic nerve responses, if the product of intensity and duration of the stimulus is constant there results a constant effect, provided short flashes are used (Hartline, 1934; Graham and Margaria, 1935). Figure 19 (from Hartline, 1934) contains records of single optic nerves from *Limulus*. The records in horizontal rows were responses to flashes of constant intensity and varying duration, those in vertical rows were responses to constant duration flashes of varying intensities. In diagonal rows the product of intensity and duration was equal; and as would be expected, the responses in diagonal rows were approximately the same. In the case of spectral sensitivity a similar parallel exists. The spectral sensitivity of a single *Limulus* ommatidium resembles the absorption spectra of the rhodopsins of many animals, as well as the human scotopic visibility curve (Graham and Hartline, 1935). With respect to dark adaptation, too, it is observed that the sensitivity change of a single ommatidium resembles the course of dark adaptation as observed in psychophysical experiments, except that in *Limulus,* with only one morphological receptor type, there is evidence of only one receptor process (Hartline and McDonald, 1947). Thus, analysis of the response of the single *Limulus* ommatidium suggests that, in general, the known properties and postulated photochemical reactions of the visual pigments are reflected in the properties of the receptors, the responses of which in turn are important in determining basic visual functions.

This is not to say, however, that vision ends with receptor cell processes. Even in the primitive compound eye of *Limulus,* there occurs neural interaction, in this case a mutual inhibitory interaction that modifies the activity of the ommatidia (Hartline, 1949; Hartline *et al.*, 1956; Hartline and Ratliff, 1957, 1958; Ratliff *et al.*, 1958; Tomita, 1958). In the retinas of higher invertebrates and vertebrates there are ganglia as complex as those in central nervous system optic centers and other parts of the brain. And in these retinas there occur both excitatory and inhibitory neural interactions. However, the response of the visual receptors provide the only means of assaying the physical stimulus and any neural alterations or modifications can add no new information; they can only accentuate or diminish various aspects of the receptors' activity.

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CHAPTER 7

Muscle Cells

By H. E. HUXLEY

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I. Introduction

A. *Structure and Function*

The existence of animal life depends on the possibility of purposeful movement. Such movement has become practicable through the development of highly specialized structures which can transform chemically stored energy into mechanical work. The character of the animal life which exists depends very largely on the speed and efficiency with which this process takes place.

In the larger animals, these contractile structures are organized into muscle fibers. The sudden and dramatic mobilization of energy in the contraction of a vertebrate skeletal muscle is such a striking and readily observable phenomenon that its nature has invited speculation since the earliest times, and it still demands explanation, or at least description, in terms of contemporary physics and chemistry. But so far, we remain almost completely ignorant of the fundamental molecular events which are involved. A very great deal of well-established information concerning many aspects of muscular contraction—physiological, biochemical, and structural—has now been built up, however; for muscle is a favorable as well as an attractive system on which to work. It will be the purpose of this chapter to review these aspects of our knowledge which seem (to the author at least) particularly relevant to a consideration of the problem in detailed molecular terms. The volume of data now available is so great that a simple, unselective, and unbiased enumeration of all of it would be tedious and incomprehensible, as well as impracticable; one needs to view it from a particular standpoint so that the facts can be arranged in some sort of pattern: so that at least they can be seen easily, and remembered, if not explained.

In the last few years, a large body of evidence has accumulated which has enabled the ultrastructure of the contractile elements in muscle to be described in terms of a simple model—the "interdigitating" or "sliding filament" model. In this article, therefore, the data will, wherever possible, be considered in their relation to this structural model. It is hoped that this procedure will bring together many of the functional and biochemical observations on muscle, and at the same time enable us to ask the right sort of questions about the detailed molecular events which produce contraction. We will describe the main features of this model, then, as soon as possible. But before we can do so, we need to set down an elementary framework of information in which it can be fitted.

B. *The Various Types of Muscle*

The skeleton muscles of vertebrates (which produce the familiar rapid movements of limbs and body) show, when examined in the light microscope, a pattern of very narrow bands which run transversely to the direction of shortening of the muscle. These muscles are said to be cross-striated; their properties are known in considerable detail and they provide a readily available and highly satisfactory experimental material. It is with this type of muscle that we shall mainly be concerned in this article, and it is to this type of muscle that the model refers. Many other muscles, however, do not display any such pattern of striations. Examples of these so-called 'smooth' muscles may be found in the muscles used to produce intestinal movement, dilation of the capillaries, contraction of the uterus; and the closing together of the two halves of the shells of mollusks. As a general rule, smooth muscles shorten more slowly than striated ones, but exceptions to this rule do exist; and muscles which could all be called "smooth" (i.e., without transverse striations) are found to differ among themselves, both in their structure and their behavior, in ways at least as striking as those in which they differ from striated muscle (Hanson and Lowy, 1957). To what extent some of the features of the structural model which has been proposed for striated muscle are also present in smooth muscles is a matter for current research.

C. The Main Histological Features of Vertebrate Striated Muscle

The muscle bundles are composed of very large numbers of approximately longitudinal muscle fibers. The diameters of the muscle fibers usually lie in the range 10–100 μ ($1 \mu = 1$ micron $= 10^{-4}$ cm.). Each muscle fiber extends for a considerable distance along the length of the muscle, and some may even extend along the whole length. Some muscles, for instance, rabbit psoas or frog sartorius, have a very parallel arrangement of fibers and are therefore particularly suitable for studies of their mechanical properties. The fibers terminate in tendonous connection to the bone or the organ which they have to move. Muscular contraction is brought about by a shortening of the fibers. *In vivo,* this takes place when an electrically polarized membrane, which surrounds each fiber, is depolarized (Kuffler, 1946; Katz, 1950).

The contractile material in the fibers is contained in the fibrils, which have a diameter of 1 or 2 μ, are very long, and are arranged parallel to the long axis of the fibers (see Fig. 1). The fibrils display a pattern of striations like the intact fiber, and usually they are arranged side by side with their striations in register. This is not always the case, however, and examples are often seen where the fibrils have been sheared past each other so that their striations are staggered. Moreover, Tiegs (1955) has shown a number of cases where helical arrangements of striations seem to be present, particularly in insect muscles. None the less, the pattern of striations in the *fibrils* is a simple transverse one, and this will be a convenient point at which to describe its main features. These are illustrated in Fig. 1. The appearance of the striations as seen in the phase contrast and polarizing light microscopes is shown in Fig. 2.

The repeating unit in this pattern is the sarcomere. This is the segment delineated by two successive narrow dark lines, the Z-lines (or the Z membranes as they are sometimes known). The length of a sarcomere is usually about 2 or 3 μ, and so many thousands of them go to form each fibril. The main feature of the resultant pattern of striations in the fibril is the alternation of successive dense, birefringent bands (the anisotropic or A-bands) and less dense, almost nonbirefringent bands (the isotropic or I-bands). The I-bands are bisected by the Z-lines, so that each A-band forms the middle portion of a sarcomere. At rest length, the central region

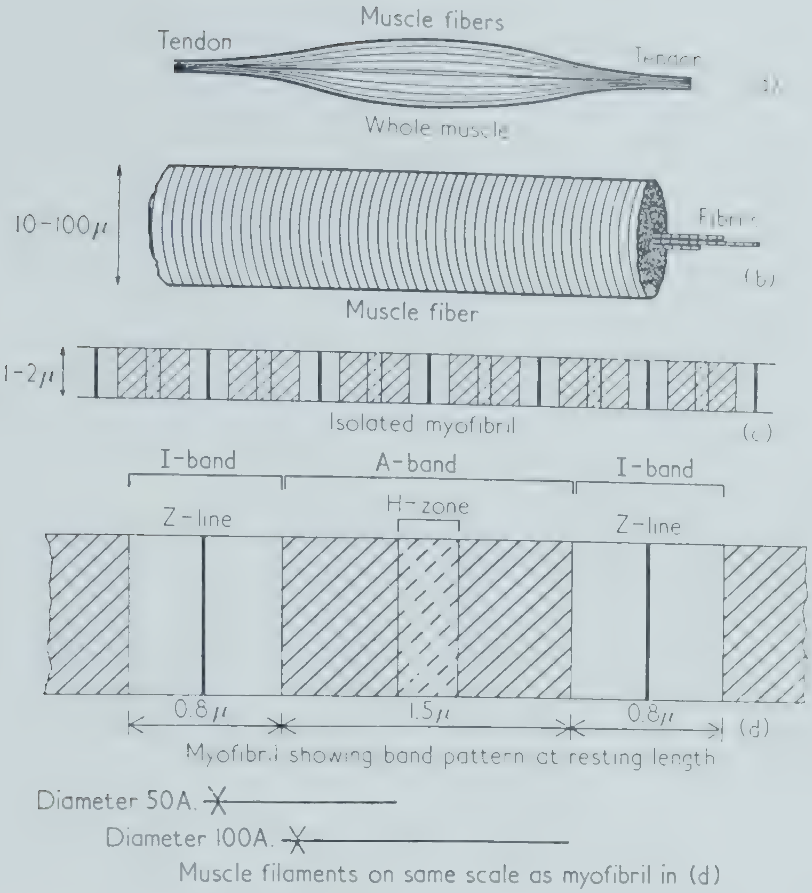

Muscle fibers
Tendon
Tendon
Whole muscle
10–100μ
Fibril
(b)
Muscle fiber
1–2μ
Isolated myofibril
(c)
I-band
A-band
I-band
H-zone
Z-line
Z-line
0.8μ
1.5μ
0.8μ
(d)
Myofibril showing band pattern at resting length
Diameter 50A.
Diameter 100A.
Muscle filaments on same scale as myofibril in (d)

FIG. 1. The structure of striated muscle at different levels of organization; dimensions shown are those for rabbit psoas muscle.

of the A-band has a slightly lower density than the rest, and is referred to as the H-zone. There are often some additional features of the cross striations visible, but we need not refer to them at this point. In the electron microscope (Fig. 3), the fibrils can be seen to be built up of parallel longitudinal filaments the diameter of which is of the general order of magnitude of 100 A. (1 A. = 10^{-8} cm., i.e., 10,000 A. = 1 micron). The band pattern must be a reflection either of the nature and arrangement of the filaments themselves, or of material lying between them. It will be seen that the former view appears to be the correct one.

The fibrils are of course by no means the only structures visible inside the muscle fibers. There are also present nuclei—a considerable number inside each fiber; mitochondria—in very large numbers in heart muscle, insect flight muscle, and others capable of continuous activity for prolonged periods; and a highly complex system of interconnected membranes or vesicles (Bennett and Porter, 1953; Porter and Palade, 1957). And it is known from biochemical studies that large amounts of soluble (as opposed to structural) proteins are also present inside the muscle

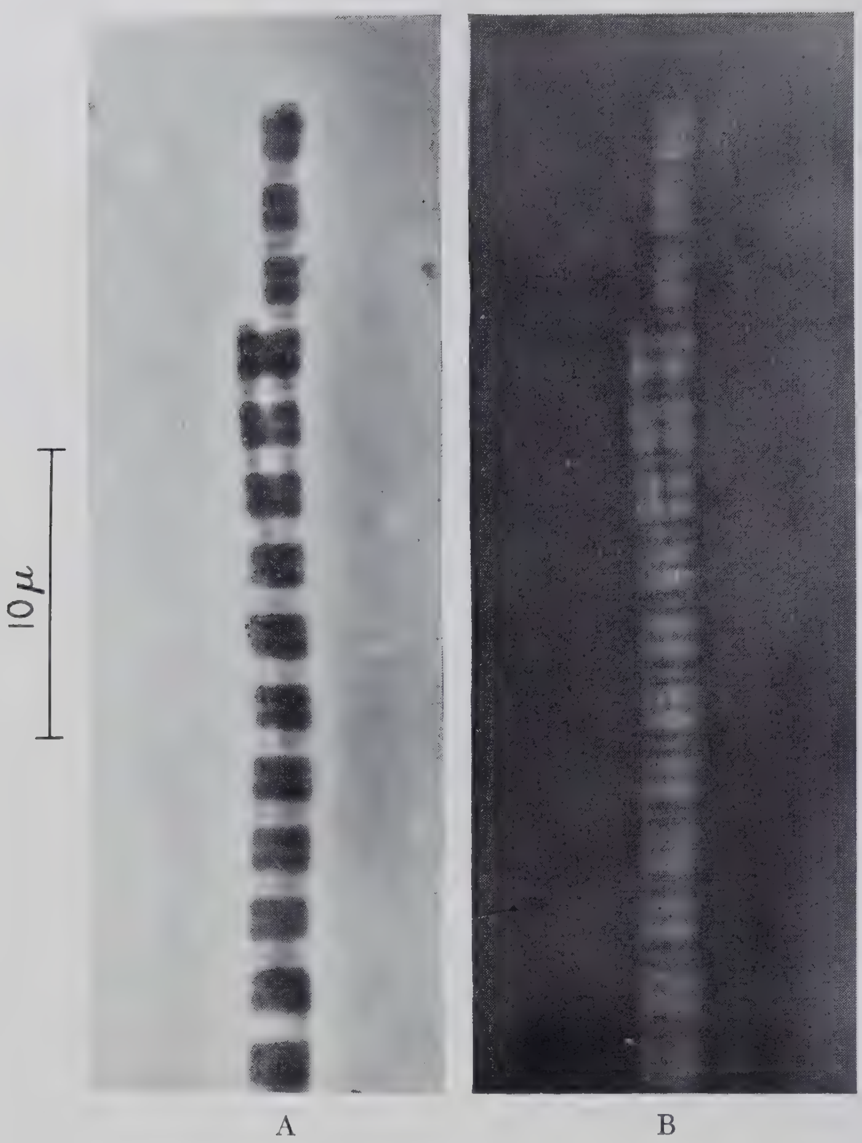


FIG. 2. Isolated myofibril from rabbit psoas muscle (glycerinated). A. In phase contrast illumination. B. In polarized light. Note correspondence between different zones, the denser bands (A-bands) being the more birefringent. (From Hanson & Huxley, 1955.)

fibers, many of them concerned in the metabolic processes associated with contraction.

D. Basic Mechanical Properties

At rest, muscle is flexible and rather plastic and may be reversibly extended by a small force. When the external membrane of the fiber is depolarized, however, a very rapid change in the muscle substance takes place, and it becomes rigid, inextensible and begins to shorten and exert tension. In frog sartorius muscle at 0° C., for example, the active state is fully developed in about 40 milliseconds after stimulation (Hill, 1949a).

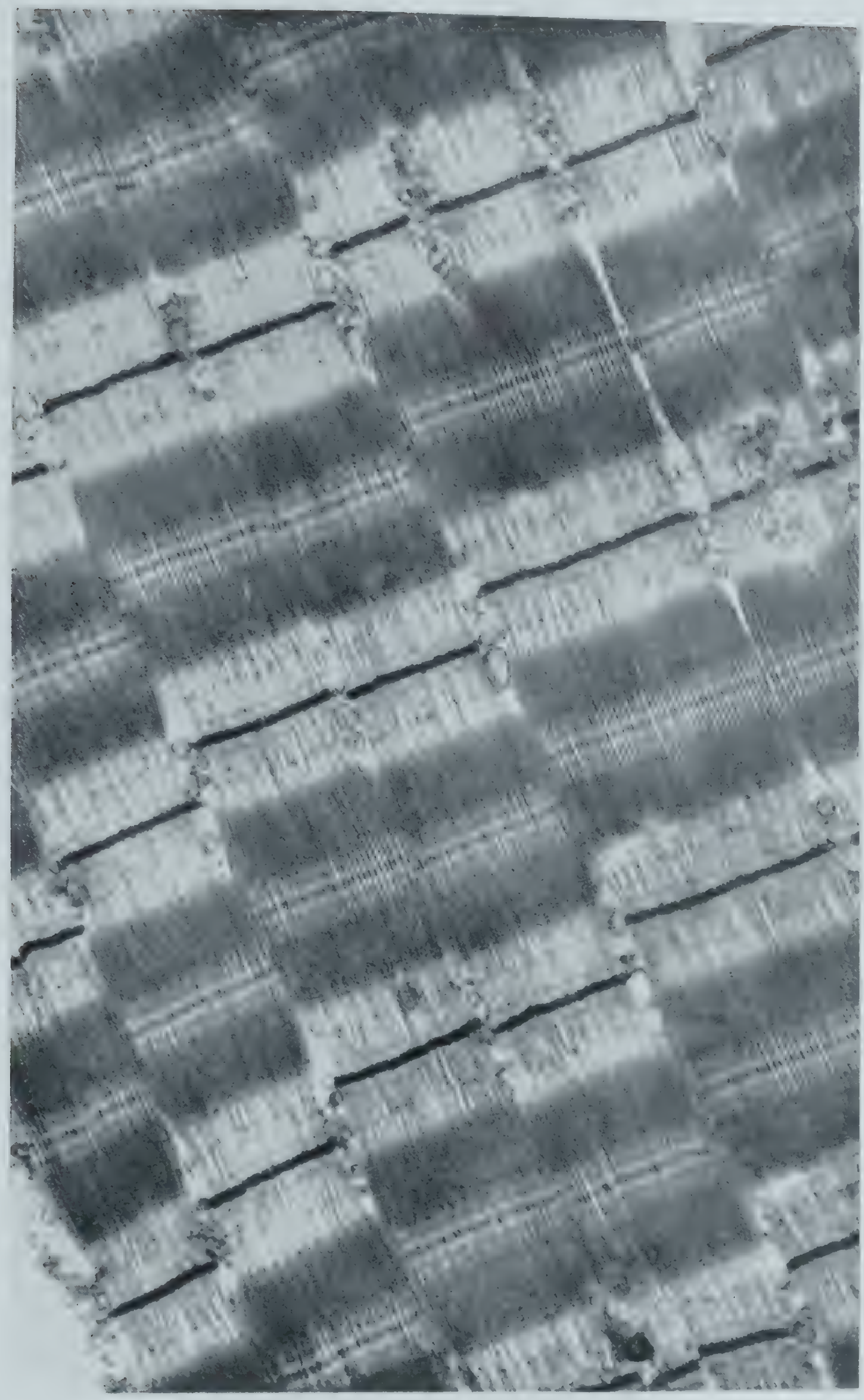


FIG. 3. Low-power electron micrograph of section of rabbit psoas muscle; the pattern of A-, I-, Z-, and H-bands may be seen much more clearly than in the light microscope pictures, and the longitudinal filaments which make up the contractile material are also visible. Granules and/or vesicles lie in between the myofibrils. Magnification: ×24,350.

If the contraction has been produced by a single traveling wave of depolarization (an action potential) passing along the fiber membrane, then the active state soon begins to decay again; in frog sartorius muscle at 0° C., the decay of activity is detectable almost as soon as it is fully developed (Macpherson and Wilkie, 1954), and the resting state is fully restored again in about a second or so. The muscle is said to have carried out a single twitch. If contraction is produced by a close succession of action potentials, then the active state is maintained; such a contraction is known as a tetanus, and this is what is normally observed *in vivo.*

During a tetanus, the skeletal muscles of vertebrates can exert a maximum tension of the order of a few kilograms per square centimeter of their cross section. Maximum tension is developed only when the muscle is prevented from shortening. If the tension applied is reduced, then the muscle will shorten with a certain *velocity.* (This behavior may be contrasted with that of a stretched spring, which would shorten with a certain *acceleration*). The velocity of shortening varies with the load in a perfectly definite manner which can be described by the well-known Hill equation (Hill, 1938):

$$(P + a)\, v = (P_0 - P)\, b$$

Where P_0 = maximum tension (at zero velocity of shortening); P = tension produced when velocity of shortening is v; a and b are constants. As the velocity of shortening is allowed to increase, the load that can be raised decreases, until, at a shortening velocity

$$v_{\text{max}} = \frac{P_0\, b}{a} \simeq 4b$$

the muscle exerts no external force at all. The order of magnitude of the maximum velocity of shortening is one to ten muscle lengths per second. Under half-maximum load, the velocity would be about six times smaller. This equation is found to apply over the whole range of muscle lengths, providing the maximum tension appropriate to that length is inserted into the formula (Abbott and Wilkie, 1953).

The maximum tension generated by a muscle is a function of the length of the muscle relative to its resting length in the body; the tension is greatest around rest length (R.L.), and is smaller at longer or shorter lengths, becoming very small indeed at about 60–65% R.L.

An active muscle liberates heat, as we all know. Part of the heat appears whether the muscle shortens or not, and this part is known as the activation heat in a twitch and the maintenance heat in a tetanus. If the muscle shortens, then additional heat is liberated, and this is called the heat of shortening. The amount of shortening heat produced by a muscle is proportional to the distance that it shortens and is independent of the load that it lifts. This is a very remarkable phenomenon, for it means that when a muscle lifts a large load, a greater total amount of energy is liberated for a given amount of shortening than when it lifts a small one. The extra energy liberated is almost exactly equal to the extra work done. This shows us straight away that we are dealing with a very interesting and subtle mechanism indeed; and that an active muscle is not just simply a body tending to move to a state of lower potential energy, like a stretched spring or a rubber band.

If a muscle is deprived of its supplies of oxidative and glycolytic energy, it soon passes into a state of rigor. It becomes rigid and inextensible, but it is no longer able to contract. There is strong evidence (Erdos, 1943; Bate-Smith and Bendall, 1947, 1949; Bendall, 1951) that this change is associated with the disappearance of adenosine triphosphate (ATP) from the muscle.

E. Main Biochemical Features

The contractile elements of striated muscle, the myofibrils, are composed very largely of protein (Perry, 1951), and the number of different proteins present in them in important amounts is relatively small. The three principal proteins present are myosin, actin, and tropomyosin. Myosin makes up about 54% of the total protein in the myofibrils (Hanson and Huxley, 1957), actin about 20% (Perry, 1953), and tropomyosin perhaps about 10–12% (Perry and Corsi, 1958). The balance of ~15% has not been characterized.

Myosin is an enzyme which can catalyze the dephosphorylation of ATP (Engelhardt and Ljubimova, 1939). This reaction is known to be very closely linked to the contractile event (Lundsguaard, 1930a, b, 1931). Actin, (Straub, 1942), will combine with myosin to form a complex, known as actomyosin (Straub, 1942, 1943a). In high salt concentrations ($\sim 0.6\,\mu$) this complex is dissociated by ATP (Straub, 1942; A. Weber, 1956). Artificial fibers can be prepared from precipitated actomyosin, and these will contract when they are immersed in a solution containing ATP and suitable salts (A. Szent-Györgi, 1942). It therefore seems that the essential contractile structure consists simply of these two proteins and that the reaction of this structure with ATP results in contraction and in the dephosphorylation of the ATP.

Myosin can be selectively extracted from rabbit muscle, leaving actin and tropomyosin behind, by solutions of high strength in the presence of ATP (or of pyrophosphate, which also dissociates actomyosin). The individual myosin molecules are quite large and probably have a molecular weight of about 400,000 or 800,000 (see Section III, A, 1, a). Soluble in high salt concentrations, they will easily aggregate to form semicrystalline sheaves when precipitated at lower salt concentrations (A. Szent-Györgyi, 1949), and they can be made into artificial fibers by a variety of techniques. Artificial fibers made from myosin alone will not contract in ATP; the presence of myosin and actin together is necessary (Hyashi, 1958).

Actin can exist in two forms, which are generally referred to as G-actin and F-actin. This terminology was introduced to take account of the fact that in low salt concentrations, actin behaves as a globular protein, with a molecular weight of $\sim$70,000 or 140,000 (Feuer *et al.*, 1948; Mommaerts, 1952a; Tsao, 1953a), whereas in the presence of higher salt concentrations it seems in many ways to behave like a typical fibrous protein, and indeed exhibits spontaneous birefringence and highly anomalous viscosity suggestive of the presence of very long polymers in solution. This led to the belief that a straightforward polymerization of the G-actin units has taken place, but recent experimental work by Tsao (1953a, b), which we will refer to in detail later, shows that a very sophisticated approach to the data may be necessary.

Tropomyosin (Bailey, 1946, 1948), too, has many of the properties associated with fibrous proteins. The monomeric units, with a molecular weight of about 53,000 (Tsao *et al.*, 1951) have an axial ratio of $\sim$26. A possible form of the molecule would consist of a double chain structure about 340 A. long containing a single cyclic polypeptide chain looped back on itself. Tropomyosin forms viscous nonthixotropic solutions which show a characteristic drop in viscosity when the salt concentration is raised to 0.05–0.1 M. So far, no interaction between tropomyosin and either myosin, actin, or actomyosin has been demonstrated.

After this brief outline of some of the properties of muscle, we can now describe the detailed structure of the contractile elements, and the general way in which changes in the length of the muscle are produced. The picture that emerges is a slightly unexpected but a simple one, and it is supported by a large number of different lines of evidence, some of them of a very direct character. This evidence will be described in some detail before we go on to consider the other properties of muscle and muscle proteins and the light they throw on the more detailed molecular processes taking place during contraction.

II. The Interdigitating Filament Model

A. *Definition*

The contractile structure of striated muscle is built up from two types of longitudinal filaments; the filaments in rabbit psoas muscle have

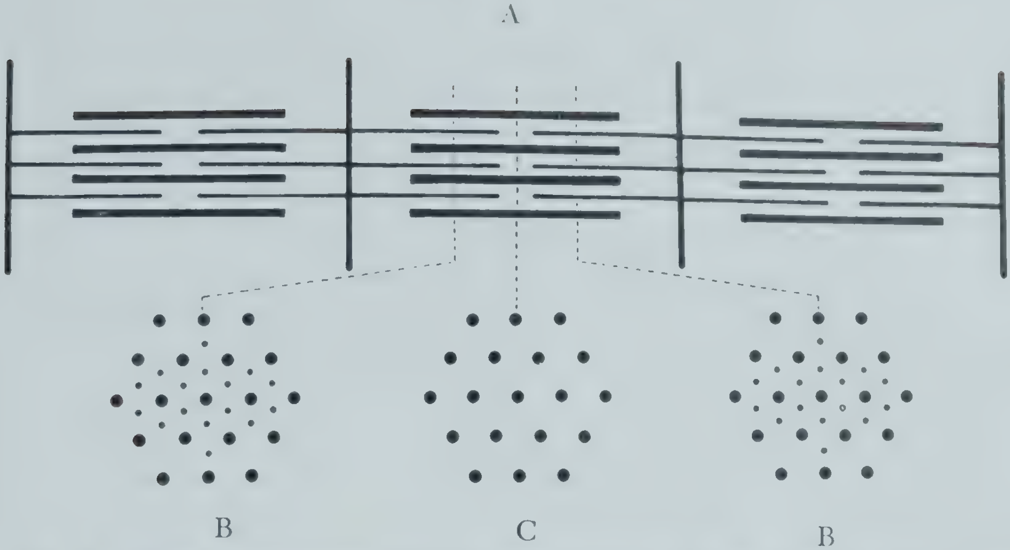


FIG. 4. The arrangement of the filaments in the fibrils of striated muscle. A. Longitudinal view, showing the series of overlapping arrays. B. Cross section through region of overlap, showing double hexagonal array. C. Cross section through H-zone, showing simple hexagonal array of large filaments only.

diameters of about 100 A. and 50 A., and lengths of 1.5 μ and 2.0 μ respectively; similar dimensions apply for frog sartorius and other vertebrate skeletal muscles. These filaments are organized into a series of discrete overlapping arrays, as shown in Fig. 4, each array containing filaments of one type only, arranged in register.

The thicker filaments extend from one end of the A-bands to the other, and they lie side by side 450 A. apart in an hexagonal array. The thin filaments extend in either direction from the Z-lines all the way to the edges of the H-zones. Thus they penetrate in between the thick filaments in the A-bands on either side of the H-zone. The ends of opposing thin filaments are possibly linked together across the H-zone by some readily extensible material.

In the region where the two sets of filaments overlap, each thick filament has around it six thin filaments which it "shares" with the six neighboring thick filaments in the hexagonal array (see Fig. 4). The thin filaments tend to occupy the trigonal points in the hexagonal lattice (i.e., they tend to lie symmetrically between three primary filaments). Thus each thin filament has *three* thick filaments around it, and so it can be seen that there will be twice as many thin filaments as thick ones. Cross bridges extend outward from the thick filaments and can link onto the adjacent thin filaments. The bridges occur on the average about every 60–70 A. along a given thick filament, so that there are six of them every 400 A., and each of the six neighboring thin filaments can form one connection to this thick filament in this distance. The thick filaments are composed largely of myosin; the thin filaments contain the actin component of the muscle, and probably also tropomyosin. The cross links between filaments represent actin-myosin linkages and correspond to the formation of the actomyosin complex.

When the muscle changes its length, either actively or passively, the thin, secondary filaments slide either farther into or farther out of the parallel array of thick primary filaments which forms the A-band, and the length of either type of filament does not change appreciably during the process. When a decrease in length takes place which is sufficient to bring the ends of adjoining arrays of thin filaments (and later of thick filaments) into apposition, then the filaments may crumple or overlap at their ends, but over the rest of their length they stay straight and the sliding process proceeds as before.

In resting muscle, the presence of ATP keeps the actin-myosin complex dissociated and the cross links are not attached to the actin filaments; the two sets of filaments can slide past each other easily and the muscle is readily extensible. In actively contracting muscle, a repetitive cyclic process goes on at each link, the links being attached to the actin filaments for one part of the cycle and disconnected for the rest. Each time one cycle of operation of the links takes place, the actin filaments are forced to slide a short distance along past the myosin filaments. During an entire contraction, each link will act a considerable number of times. Although for a good many purposes it will not be absolutely necessary to specify the manner in which the cross links operate any more closely than this, it will be more satisfactory if a few suggestions are made here concerning their possible detailed behavior; and these possibilities can be borne in mind when evidence touching on them comes to be discussed.

There are broadly two ways in which such a system might operate (H. E. Huxley and Hanson, 1954; A. F. Huxley and Niedergerke, 1954; Hanson and Huxley, 1955). The more straightforward one supposes that a repetitive change in the length (if they are obliquely oriented) or in the position of the cross links takes place, in such a way that during the part of their cycle when they are attached to the actin filaments they exert a tension and draw those filaments along. During the other part of the cycle they detach from the actin, return to their previous configuration, and attach themselves to the actin filament at a new point. The cycle then repeats itself. Change in position of the links might occur as a result of the binding or the splitting of ATP; or as a result of thermal motion (A. F. Huxley, 1957). This repetitive making and breaking of connections would correspond to actin-myosin complex formation followed by the dissociation of the complex by ATP, which is then split and discarded by the myosin. The essence of the system is that each link exerts a tension while it moves an appreciable distance (~50–100 A.) during each cycle of its operation, and that these tensions add together in parallel. The total movement of the actin filament is determined by the total "rectified" movement of any given link, and the total tension is the sum of the tensions generated at all the links.

Another way in which such a system might operate is as follows. Let us suppose that the axial repeating period in the actin filaments is slightly less than that of the myosin filaments. Let us also suppose that when combination takes place between the actin and myosin, via the cross links, the forces involved are sufficient to stretch or to break up the actin filaments; and that when the actin is dissociated from the myosin again by the arrival of more molecules of ATP, the actin filaments revert to their normal condition and length. If we start off with the actin and myosin combined, and detach the actin filaments progressively from the ends of the A-bands inward, then the actin filament will be slowly drawn in as it shortens to its "free length"; until the next site along the attachment to myosin in the actin filament comes near to the first cross bridge which we detached. We now suppose that a wave of reattachment now takes place until the system reverts to its original condition—with the difference now that the actin filament has moved along by one attachment period. In this system, the length changes produced by all the links add together, and each need only be a few Angström units.

B. *Direct Evidence for the Interdigitating Filament Model*

This evidence may be considered in two parts. On the one hand there is the structural evidence, some of it very direct, some of it less so; on the other, a number of pieces of supporting evidence, which give powerful indications of the possible structures and mechanisms which could be present. We will take the structural evidence first.

1. X-Ray Diffraction Evidence

X-Ray diffraction patterns give evidence about structural regularities in the range 1–1000 A. Where these patterns are very detailed, and where the actual repeating unit is not too complicated, the structure can usually be solved completely and a position assigned to each individual atom. In the case of crystalline proteins, the pattern is still sufficiently detailed to enable the structure to be solved "in principle," but in practice the problem is one of stupendous difficulty. In the case of muscle, the X-ray diagram is very lacking in detail in comparison with that given by a protein crystal, and the complexity of the structure is even greater. However, limited though it is, the diffraction data have given some information, and a number of very important clues, about the striated muscle system.

a. Wide-angle pattern. Muscle gives a wide-angle X-ray diffraction pattern of the α-type (Herzog and Janke, 1926; Astbury and Dickinson, 1940); that is to say, there are reflections which would correspond to a repeating periodicity of 5.1 A. along the axis of the muscle and one of 10.5 A. at right angles to the long axis. This type of structure is characteristic of polypeptide chains in the so-called α-helix configuration (Pauling *et al.*, 1951; Pauling and Corey, 1951). Moreover, muscle also shows a 1.5 A. axial reflection (Perutz, 1951; H. E. Huxley and Perutz, 1951) providing further evidence for the present of α-helixes oriented parallel to the long axis of the muscle. Oriented samples of actomyosin give a similar α-protein X-ray diagram (Boehm and Weber, 1932; Astbury and Dickinson, 1935a, b, 1940), while oriented actin gives a different pattern (Astbury *et al.*, 1947; Cohen and Hanson, 1956). Thus it seems likely that the myosin molecules are present in muscle in an oriented form, though the possible contribution from tropomyosin, which also gives an α-type pattern (Astbury *et al.*, 1948), should not be overlooked. It has been shown by Astbury and Dickinson (Astbury, 1947) that the α-diagram from muscle remains unchanged (apart from rather small differences in the perfection of orientation) during passive stretch, and that it is still present in muscle which has contracted. Ashbury concluded from these observations that the parts of the muscle where the polypeptide chains were well ordered, and contributed most strongly to the α-pattern, were less involved in the contractile process (assumed to be a supercoiling of polypeptide chains) than the less well-ordered regions. Pursuing the same argument further, one can say that there is no evidence for *any* change in the polypeptide-chain configuration in most of the protein in muscle during either stretch or contraction. Such constancy of configuration accords very well with a sliding filament model. We may note in passing that no change from an α to a β (extended) polypeptide chain configuration takes place in muscle unless it is *dried* before being stretched.

b. Low-angle pattern; axial spacing. On the meridian of this pattern, living frog sartorius and rabbit psoas muscle give a series of sharp reflections corresponding to an axial periodicity of about 415 A. (H. E. Huxley, 1952, 1953a) as shown in Fig. 5, A. At wider angles other reflections occur suggesting a subperiod of 54 A., but they are rather obscured by diffuse scattering unless the specimen is dried. This latter pattern has been observed by Bear (1945) in dried muscle and by Astbury *et al.* (1947) in an oriented sample of purified actin; the latter observation has recently

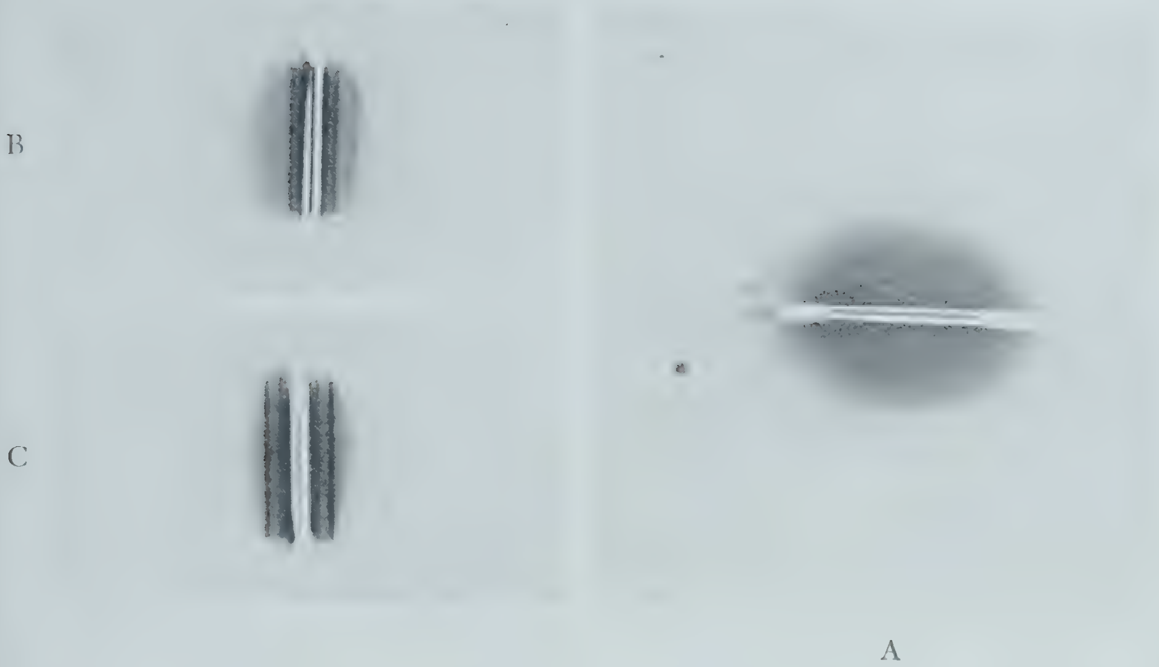


FIG. 5. Low-angle X-ray diffraction diagrams from muscle. A. Axial reflections from living frog sartorius muscle, showing presence of 415 A. repeating periodicity along the length of the muscle. B. Transverse reflections from living frog sartorius muscle, indicating presence of hexagonal array with a side spacing of 450 A. C. Transverse reflections from muscle in rigor, showing same lattice spacing but large change in relative intensities of reflections.

been confirmed and extended by Cohen and Hanson, and the spacings observed by the different authors are listed in Table I.

Many electron-micrographs of striated muscle show an approximately 400 A. axial periodicity within the myofibrils in both A- and I-bands (Draper and Hodge, 1949; Hodge *et al.*, 1954a), and so it seems reasonable to assume that the X-ray reflections arise from the contractile material.

When a living muscle is stretched, the low-angle pattern (415 A. period) remains unchanged, both in spacing and in intensity (H. E. Huxley, 1952, 1953a). No changes are observed, either, in the wider angle pattern obtained from muscle dried in the stretched condition. In a shortened muscle too, although the low angle pattern observed has deteriorated in quality, no change in the periodicity seems to take place. One must conclude from these observations that the contractile elements in muscle contain a highly ordered protein structure built up with a very precisely repeating axial period which remains unchanged after changes in the length of the muscle have taken place. It is clear that part of this regular structure must be due to actin, and so we can conclude that the periodicity of the actin polymers or filaments does not vary with the length of the muscle. It is not clear what contribution myosin

TABLE I

Meridional X-Ray Reflections from Actin and from Muscle

Oriented actin (dry) (Astbury, *et al.*, 1947) A.	Oriented actin (dry) (Cohen and Hanson, 1956) A.	Dried muscle (Bear, 1945) A.	Living muscle (H. E. Huxley, 1952) A.
—	—	—	410 ±20
—	—	—	210 ±10
—	—	—	136 ± 2
—	—	—	105 ± 5
—	—	—	83 ± 5
—	—	—	67 ± 3
—	—	58	59.3± 0.1
—	—	51	51 ± 2
—	—	—	45 ± 2
—	—	—	37 ± 1
26.8	26.7	27.2	28 ± 0.5
18.1	18.3	18.7	18.8± 0.4
13.5	13.4	13.8	13.5± 0.3
10.9	10.9	11.2	11.5± 0.3
9.1	9.0	9.2	9.3± 0.1
7.9	7.7	—	7.9± 0.2
6.8	6.8	6.9	6.95± 0.2
			(5.1± 0.05)

makes to the low-angle X-ray pattern of whole muscle, and so far no low-angle reflections have been obtained from oriented samples of myosin. As far as our information about actin is concerned, however, the low-angle X-ray data give excellent support to the idea that the actin filaments slide rather than coil and uncoil when the muscle changes its length; and indeed, the X-ray data were originally interpreted along these lines, although in terms of a somewhat different model (H. E. Huxley, 1952).

c. Low-angle pattern: transverse spacings. Living muscle also gives a few fairly sharp equatorial X-ray reflections (i.e., in a direction transverse to the fiber axis) which correspond to the pattern which would be given by a hexagonal array of long filaments 450 A. apart (H. E. Huxley, 1951, 1952, 1953a). When the muscle passes into rigor, the spacing of these reflections remains unchanged. However, very striking changes do take place in their relative intensities (see Fig. 5, B). The $11\bar{2}0$ reflection (at a spacing of 225 A.) now becomes much stronger. It is not possible to interpret this change unambiguously, but one explanation could be that some material had "crystallized out" at the trigonal points of the lattice;

i.e., that material, originally located among the main hexagonal array of filaments in a fairly random manner, had become fixed in position at points lying symmetrically between each three main filaments. As rigor is associated with the disappearance of ATP from the muscle (Erdos, 1943; Bate-Smith and Bendall, 1947, 1949), and as actin and myosin combine in the absence of ATP, it seemed likely that this change in structure was caused by actin-myosin combination. The idea was therefore put forward that actin and myosin are present in muscle in separate filaments, the myosin filaments probably forming the more permanent array with the actin filaments lying between them; in live, relaxed muscle, the actin filaments are not cross-linked to the myosin filaments, and so are not fixed in specific positions in the array of myosin filaments; when the muscle is in rigor, cross links are formed between the myosin and the actin filaments, which hold the latter fixed in the appropriate positions to give rise to the observed changes in the X-ray diagram.

This interpretation has remained perfectly valid, and it provides very strong support for the interdigitating filament model. In fact, all the X-ray data are in excellent agreement with this model. This agreement is perhaps more significant since the X-ray data were not fitted to the other observations *post hoc*, but were in fact used to predict, in advance of the other evidence, the following features of the model.

(1) The location of actin and myosin in separate filaments.

(2) The details of the double hexagonal array.

(3) The absence of any coiling or uncoiling of the actin filaments during changes in the length of the muscle.

(4) The idea that changes in the elastic properties of muscle between the resting state, activity, and rigor were due to the formation of cross linkages between the two separate sets of filaments, which altered the extensibility of the structure.

With the electron microscope and phase contrast data, these features fell into place.

The X-ray observations are also of considerable value since they enable us to feel much more confident that what we see in fixed and stained tissue in the electron microscope is a reasonably true representation of the protein structure present in the living muscle.

2. *Electron Microscope Observations*

a. Earlier work. Electron microscope investigations carried out before the era of ultrathin sectioning had revealed a number of important features about the structure of striated muscle (Hall *et al.*, 1946; Draper and Hodge, 1949). In the first place, these earlier studies, made on muscle which had been mechanically fragmented into pieces sufficiently thin for electron microscopic observation, provided convincing evidence that the detailed system of bands described by the classical light microscopists did correspond with reality and were not the results of optical artifact. Secondly, they showed that the structure was built up of longitudinal filaments having diameters in the range 50–250 A., which seemed to extend continuously over considerable distances in the muscle and which were recognized as the ultimate contractile elements. Thirdly, they showed that these filaments remained straight in muscle that had contracted; thus contraction was not brought about by any large-scale coiling or disorientation of the filaments. At the time, this observation seemed to provide support for the idea that contraction was brought about by some internal shortening of the filaments; but it is clear now that the observation is equally compatible with a sliding mechanism.

Because of the nature of the technique, in which muscle fibrils, liberated mechanically in a Waring Blendor, were allowed to dry down onto the electron microscope grid, a good many of the original structural relationships in the muscle were not preserved. The dried fibrils often appeared very flat, and it was frequently suggested that they had originally been tubular structures with the filaments at the periphery. This conclusion was not a valid one, however, for surface tension forces exert enormous pressures on small objects like these during a drying process, and *any* arrangement of filaments would be flattened very effectively. During the drying process, too, water amounting to about 80% of the total weight of the whole muscle is lost from between the filaments, which then adhere to each other in small clumps; any more subtle structural relations are lost.

b. Observations using sectioned material. To obtain a clear picture of the detailed organization of the filaments, it is necessary to work with material in which the original structure has been protected against the sort of artifact described above. This became technically possible in the early 1950's, with the development of satisfactory techniques for fixation (Palade, 1952), embedding (Newman *et al.*, 1949) and ultra-thin sectioning (Pease and Baker, 1948; Sjöstrand, 1953; Porter and Blum, 1953; Hodge *et al.*, 1954b). The first result which emerged from the application of this technique (Morgan *et al.*, 1950; Hodge *et al.*, 1954a) was that the filaments did indeed form a continuous hexagonal array across the fibrils, as indicated by the X-ray results. Further studies with improved techniques were able to demonstrate more detailed aspects of the structure (H. E. Huxley, 1953b).

When thin cross sections which had passed anywhere through the A-bands of the muscle are examined, a very regular array of filaments about 100–110 A. in diameter and 250–350 A. apart is seen (the difference between this spacing and the 450-A. X-ray spacing is probably to be accounted for by shrinkage during fixation and embedding). If the section has passed through the H-zone (the lighter region in the center of the A-band), the this simple hexagonal array is seen on its own (Fig. 6, A). However, if the section has passed through any other part of the A-band, then a second array of filaments is also seen to be present, as well as the primary hexagonal array (see Fig. 5, B). These secondary filaments, whose diameter is 50–60 A., appear at points equidistant from three primary filaments, so that each primary filament has six secondary filaments around it, which it shares with its six neighboring primary filaments. In places, bridges can be seen connecting primary and secondary filaments. If the section has passed through an I-band, however, only the thinner filaments are seen (Fig. 5, C). These electron microscope observations, together with certain crucial light microscope observations which we will describe presently, enabled a straightforward description of the structure of striated muscle in terms of the interdigitating filament model to be given (Hanson and Huxley, 1953). It was supposed that the thin filaments seen in the I-bands were continuous with the secondary array of filaments in the A-band and that they therefore extended from the Z-lines to the edge of the H-zone; and that each of the thick filaments extended continuously from one end of the A-band to the other. This arrangement, which has been shown diagrammatically in Fig. 4, accounts in a straightforward way for the appearance of the electron microscope cross sections. And as was pointed out by one of us at this time (H. E. Huxley, 1953b), the earlier ideas about the extensibility behavior of muscle and the constancy of the axial X-ray spacing found immediate expression in this model in terms of a sliding filament mechanism.

c. Observations on very thin sections. Although the double array of filaments can be seen with such clarity in cross sections, longitudinal sections do not immediately give such a straightforward picture of the double array of filaments, although of course one can see the pattern of bands which results from this overlapping system. Indeed, this is what one might expect, for in a cross section, this structure remains practically the same for the whole thickness of the section; the projected view of a slab of material is virtually the same as that of an infinitely thin plane. This is not true of longitudinal sections, however, as consideration of the three-dimensional structure will make plain. It is clear that the appearance of a parallel array of two different types of filament is bound to appear confused in longitudinal sections which contain several layers of filaments. In the electron microscope, we shall see not the real structure, but a one-dimensional moiré pattern formed by it. This effect has resulted in a good deal of controversy in the recent literature (Spiro, 1956a, b; Hodge,

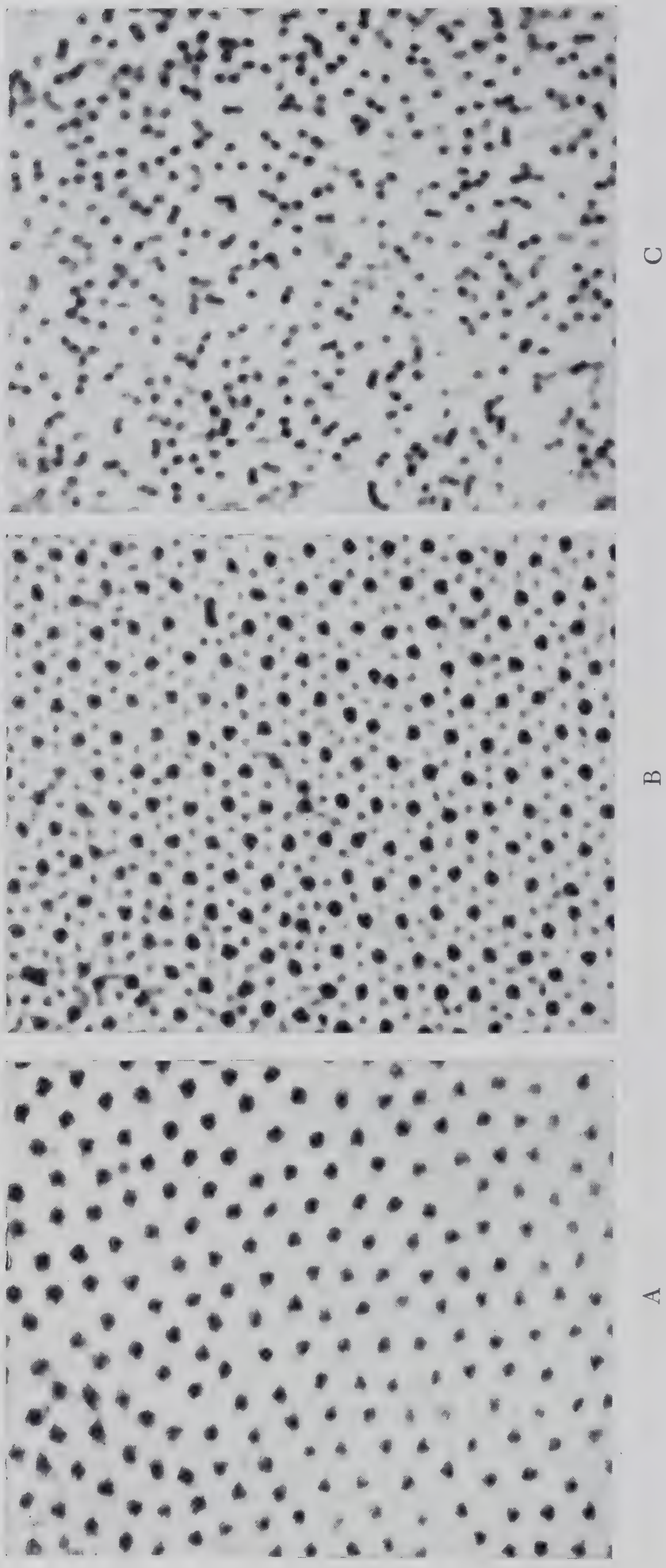
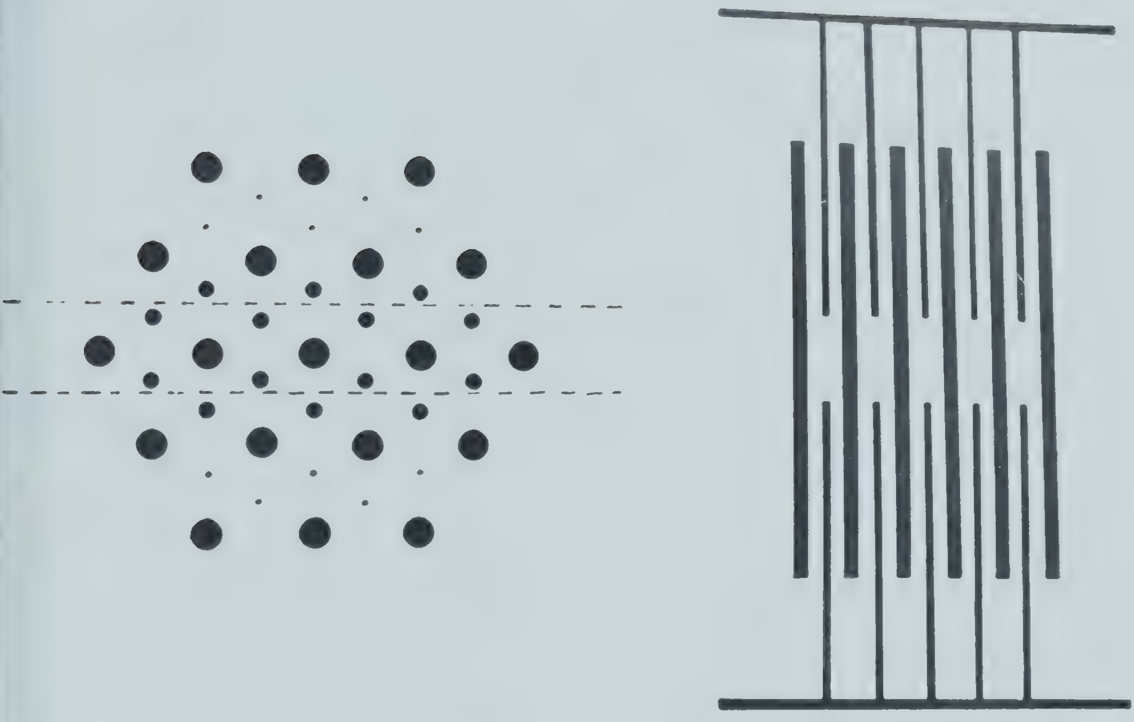


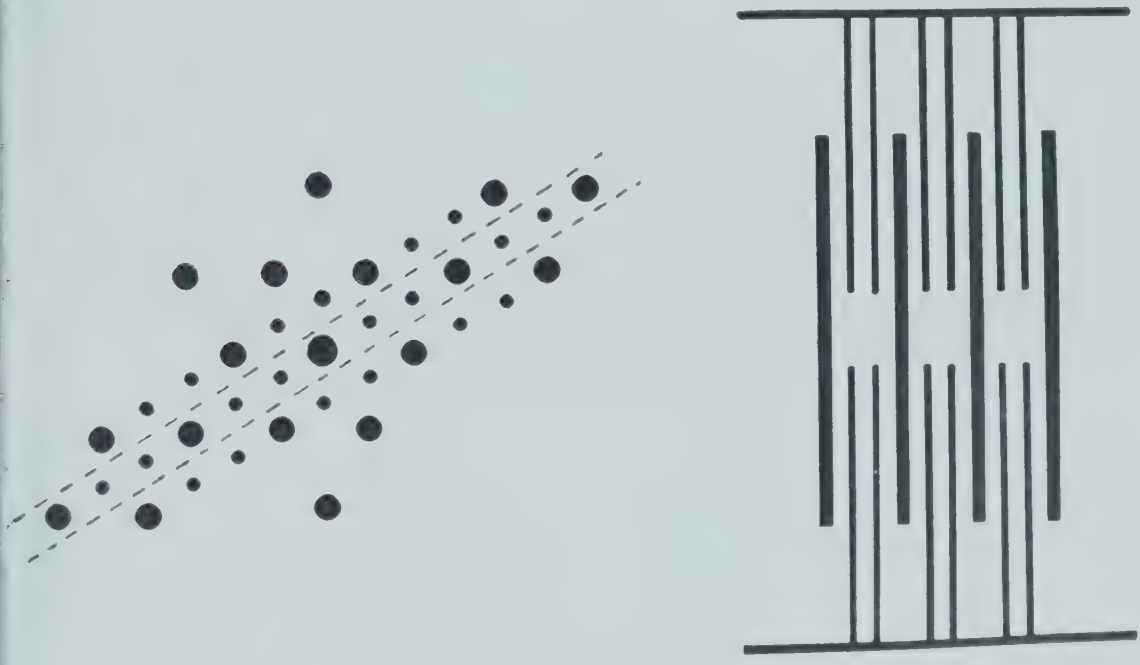
FIG. 6. Electron micrographs of cross sections of rabbit psoas muscle. A. The simple hexagonal array of thick filaments in the H-zone. B. The double hexagonal array of thick and thin filaments elsewhere in the A-band. C. The thin filament alone in the I-band. Magnification: ×150,000.



A I

A II

Fig. 7. Two possible ways of cutting ultrathin longitudinal sections through lattice of myofilaments so that there is only one layer of thick filaments lying within the section. A I. End-on view, showing longitudinal section enclosed between dotted lines 250 A. apart parallel to $10\bar{1}0$ crystallographic direction. A II. Theoretical appearance of such a section in the electron microscope. B I. End-on view, showing longitudinal section enclosed between dotted lines 150 A. apart parallel to $11\bar{2}0$ crystallographic direction. B II. Theoretical appearance of such a section in the electron microscope.



B I

B II

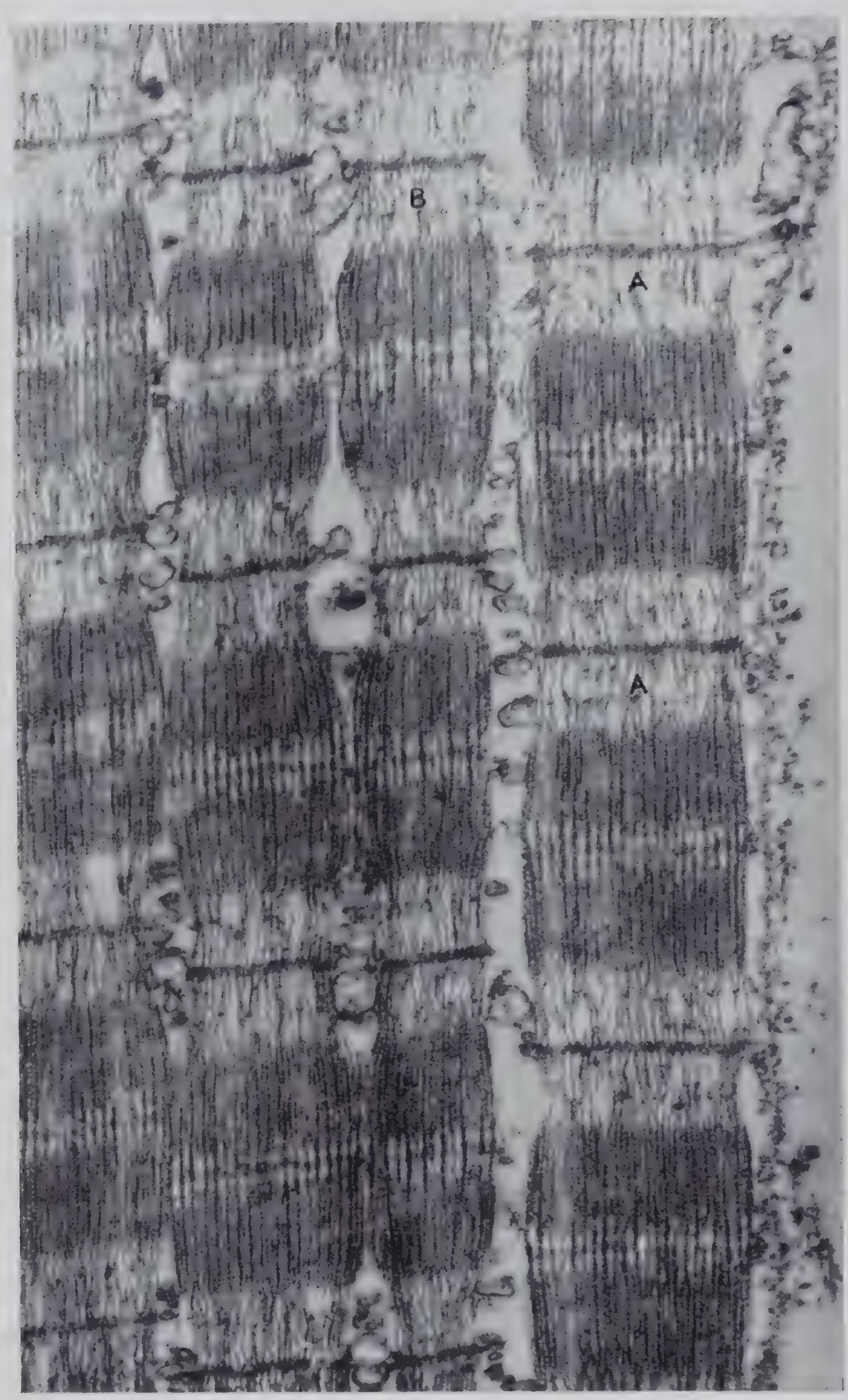


FIG. 8. Electron micrograph of ultrathin section through part of a muscle fiber; the hexagonal arrays of filaments in different myofibrils and in different sarcomeres have their axes oriented in a variety of directions; some (marked *A*) correspond to the case illustrated in Fig. 7, A; the one marked *B* corresponds to the case illustrated in 7, B. Magnification: ×42,700.

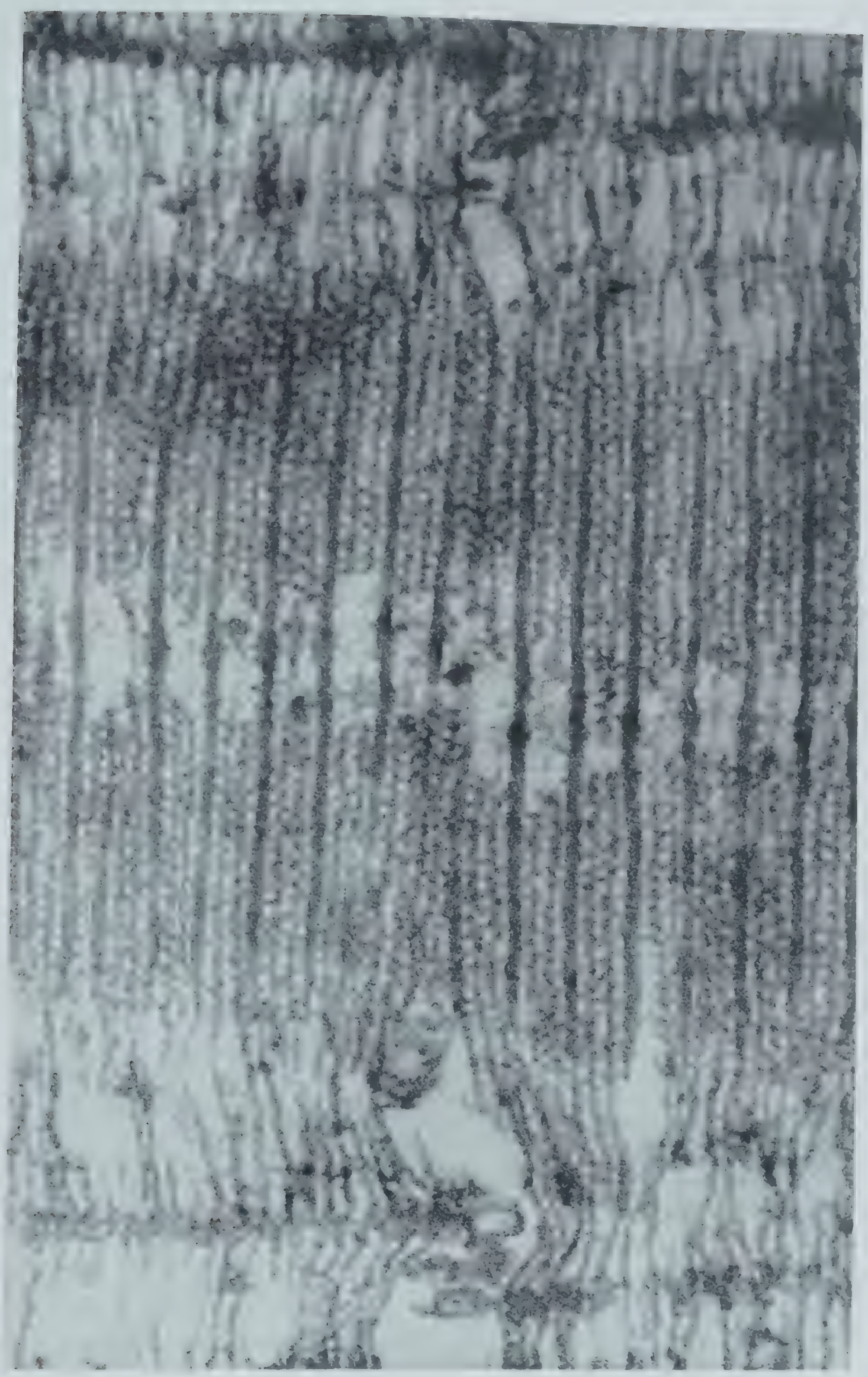


FIG. 9. Electron micrograph of one sarcomere of striated muscle myofibril (glycerinated rabbit psoas muscle) showing ultrathin section parallel to $11\bar{2}0$ direction (as in Fig. 7, B). The thick filaments extend from one end of the A-band to the other. The pairs of thin filaments between them terminate at the edges of the H-zone. Magnification: ×128,500.

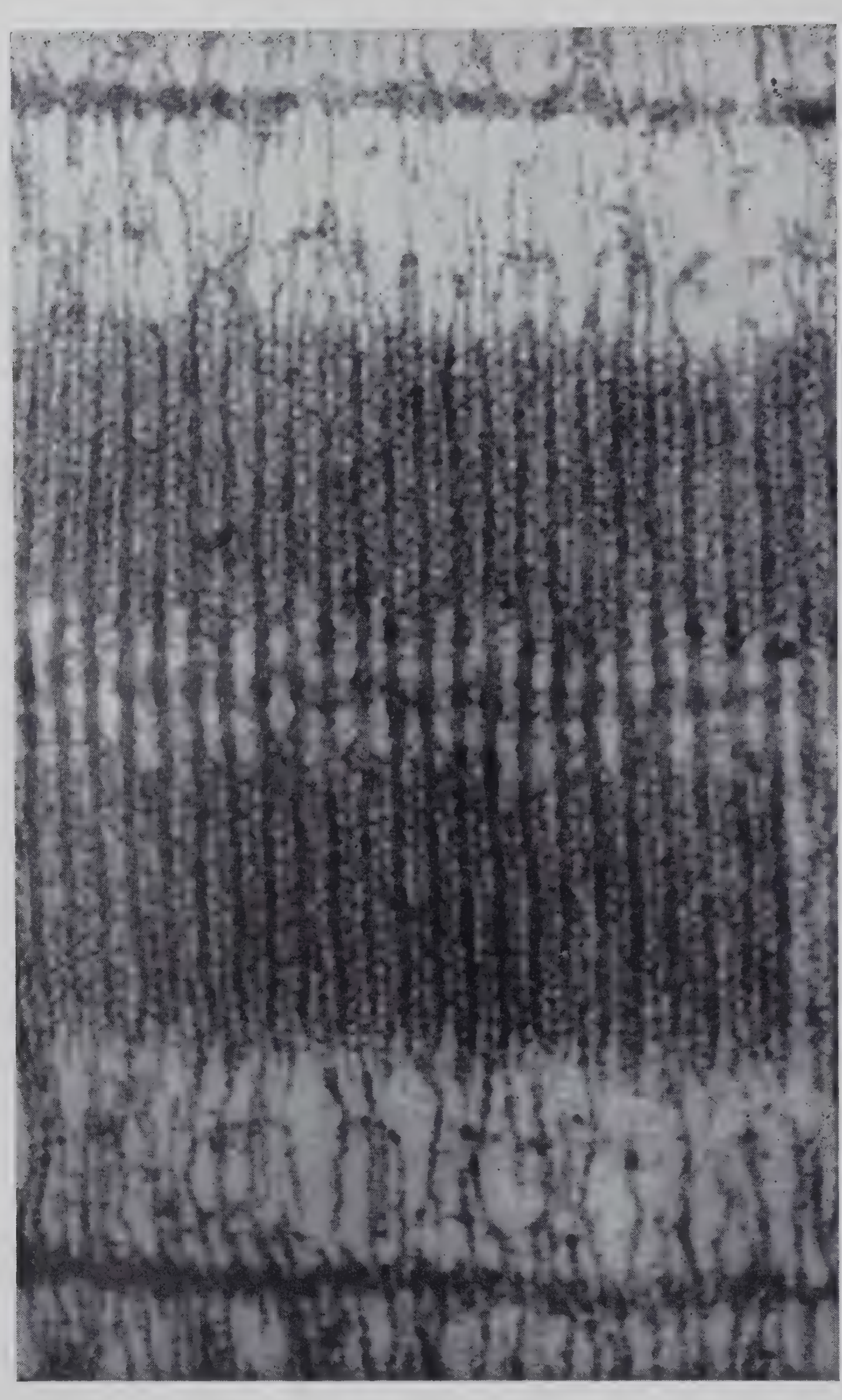


Fig. 10. As in Fig. 9, cut parallel to $10\bar{1}0$ direction. The 'thin' filaments seen here will in general correspond to two thin filaments lying in register on top of each other in the thickness of the section. Magnification: ×111,000.

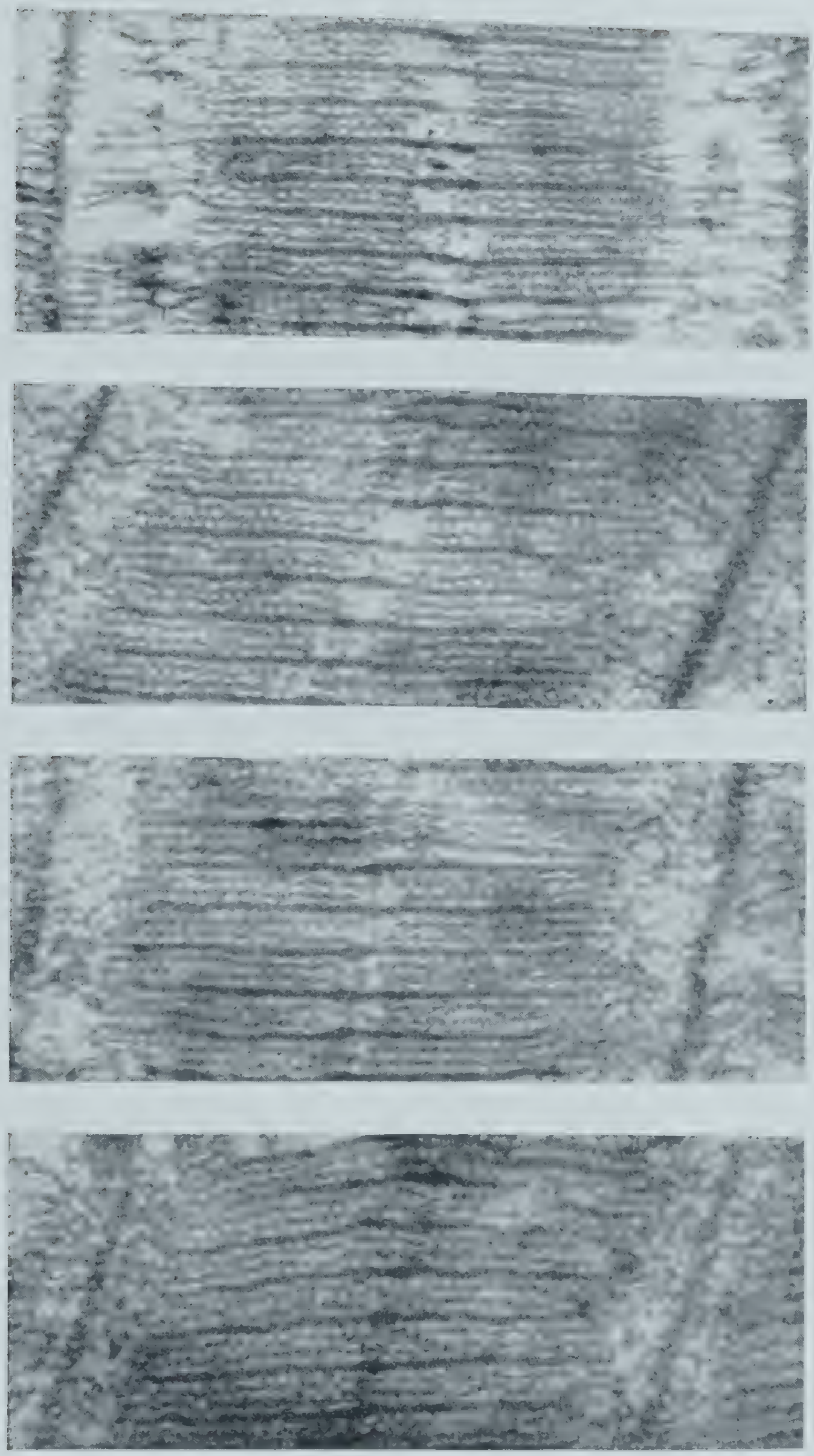


Fig. 11. Electron micrographs showing change in the degree of overlap of the two sets of filaments (and in the length of the H-zone) at different muscle lengths. Note the way the secondary filaments come together in the center of the H-zone in the slightly contracted specimen ($\sim 85\%$ rest length). Magnification: ×73,000.

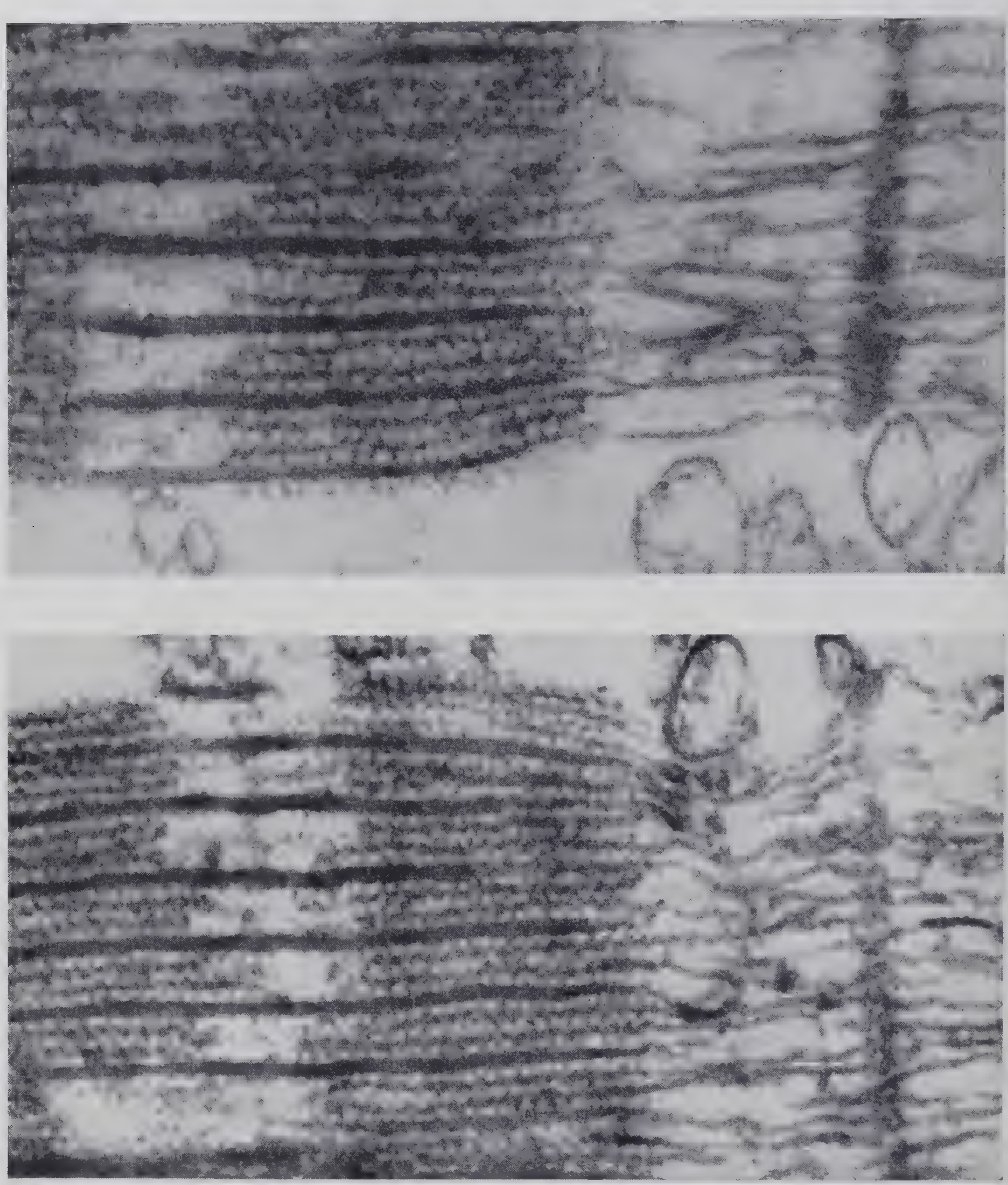


FIG. 12. Sections of muscle parallel to $11\bar{2}0$ direction, showing in some instances the tapering and the termination of the primary filaments at the end of the A-band. In the upper picture, the cross bridges protruding blindly into the sarcoplasmic space from the outermost primary filament can be seen. The termination of the secondary filaments at the H-zone, and the cross bridges between primary and secondary filaments, are also visible. Magnification: ×139,000.

1956; Sjöstrand and Andersson, 1956). These difficulties can be avoided if sections containing only one layer of filaments are used (H. E. Huxley, 1957). As the arrangement of the filaments is so regular, it is possible to cut sections which pass through a given layer of filaments over quite a substantial area. Two suitable ways in which this can be done are illustrated in Fig. 7, which also shows the theoretical appearance of longitudinal sections of muscle cut in this way.

The appearance of the tissue in suitably thin sections is shown in Figs. 8, 9, and 10, which display most of the features one would anticipate from the interdigitating filament model. The two types of filaments can be seen very clearly in the A-bands, with the secondary filaments terminating at the edges of the H-zones, the primary filaments extending from one end of the A-bands to the other, and cross bridges between them. It is found that muscles fixed at different lengths simply differ in the extent of overlap of the two sets of filaments; so that the opposing ends of the secondary filaments on either side of the H-zone are different distances apart at different muscle lengths (Fig. 11).

Although the electron micrographs show very clearly the two sets of filaments lying in their expected positions in the A-band itself, they are less satisfactorily portrayed at the ends of the A-band. Ideally, it would be desirable to see whether the primary filaments all terminate there, and whether the secondary filaments all continue on into the I-band and up to the Z-line. Unfortunately various factors militate against this, although it can be seen in some cases (Fig. 12). The degree of order of the structure is very much lower in the I-bands than in the A-bands, probably because the A-band structure is a close-packed one held together by cross links between the filaments, whereas in the I-bands the filaments are thinner, farther apart, and do not seem to have cross links between them. As a result, the filaments in the I-bands do not remain in the plane of the section for more than a short part of their course, and this disorder affects the appearance at the A-I boundary too. The thick filaments do not end squarely there, like the blunt end of a pencil, but are tapered over the last two or three thousand Angströms of their length, and in this region they often become displaced by the disorder farther on in the I-bands.

It is possible to obtain fairly direct evidence on this issue in a rather different way. If we cut serial cross sections through a given fibril, we can count the number of primary and secondary filaments in an A-band and compare these figures with the number of thin filaments in the adjoining I-band (because of the change in diameter of fibrils between A- and I-bands which often occurs, this measurement cannot be made simply by counting filaments per unit area in different bands in different

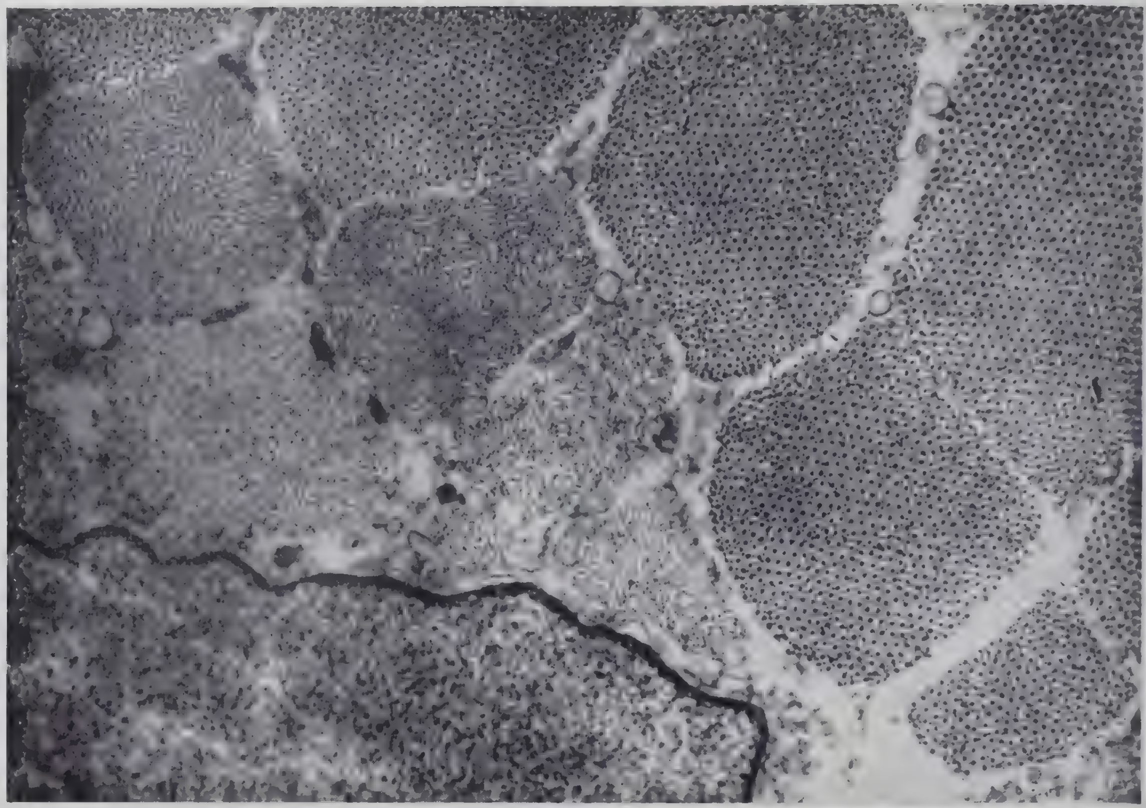


Fig. 13 A

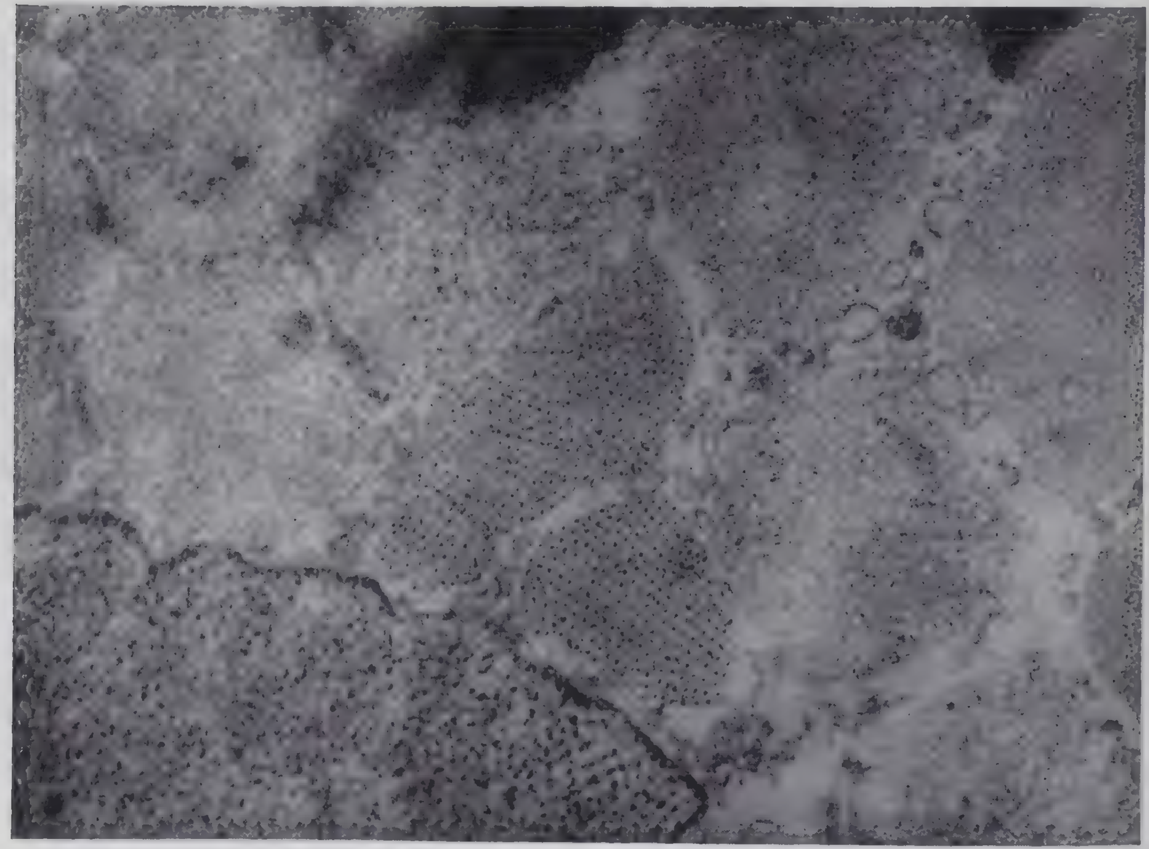


Fig. 13 B

Fig. 13. Two cross sections (about 0.6 μ apart) through a group of myofibrils in which the number of filaments present in the same fibril in different regions of the sarcomere may be counted. Filament counts on these and on other such pairs of sections are given in Table II. Magnification: ×32,000.

TABLE II

Filament Counts in Neighbouring Sections through the Same Fibril

	Fibril Number					
	1	2	3	4	5	6
No. of thick filaments in A-band	235	750	235	55	213	526
No. of thin filaments in A-band	448	1461	449	104	391	1018
No. of filaments in I-band	465	1473	461	108	388	936

fibrils). A pair of such sections is shown in Fig. 13, and Table II lists a number of sets of filament counts. It will be seen that the number of filaments in the I-bands agrees closely with the number of secondary filaments in the A-bands, and is approximately twice as great as the number of primary filaments in the A-band, as we would expect from the nature of the array. This result provides very strong support for the view that it is the primary filaments which terminate, and the secondary ones which carry on across the I-band to the Z-line.

d. The arrangement of the cross bridges. A very prominent feature of the structures seen in the thin longitudinal sections is the system of cross bridges (Fig. 14) postulated from the original X-ray studies and already observed in the cross sections seen in the electron microscope. These bridges extend from primary to secondary filaments only, i.e., they are not found between two primary filaments nor between two secondary filaments. The bridges which sometimes appear between two secondary filaments lying side by side in longitudinal sections are probably the bridges from the primary filaments lying above and below the plane of sectioning; in cross sections, bridges between secondary filaments are never seen. These bridges seem to form part of the primary filaments; where there is a primary filament lying on the very outside of a fibril (as in Fig. 12), bridges are often seen protruding from it and ending blindly in the sarcoplasm. In stretched sarcomeres, bridges still project outward from the primary filaments in the wide H-zone from which the secondary filaments have been withdrawn. The thin filaments seen in the I-bands have fairly smooth outlines and show no sign of a system of projections on them, even in the very long I-bands of stretched muscle. The arrange-

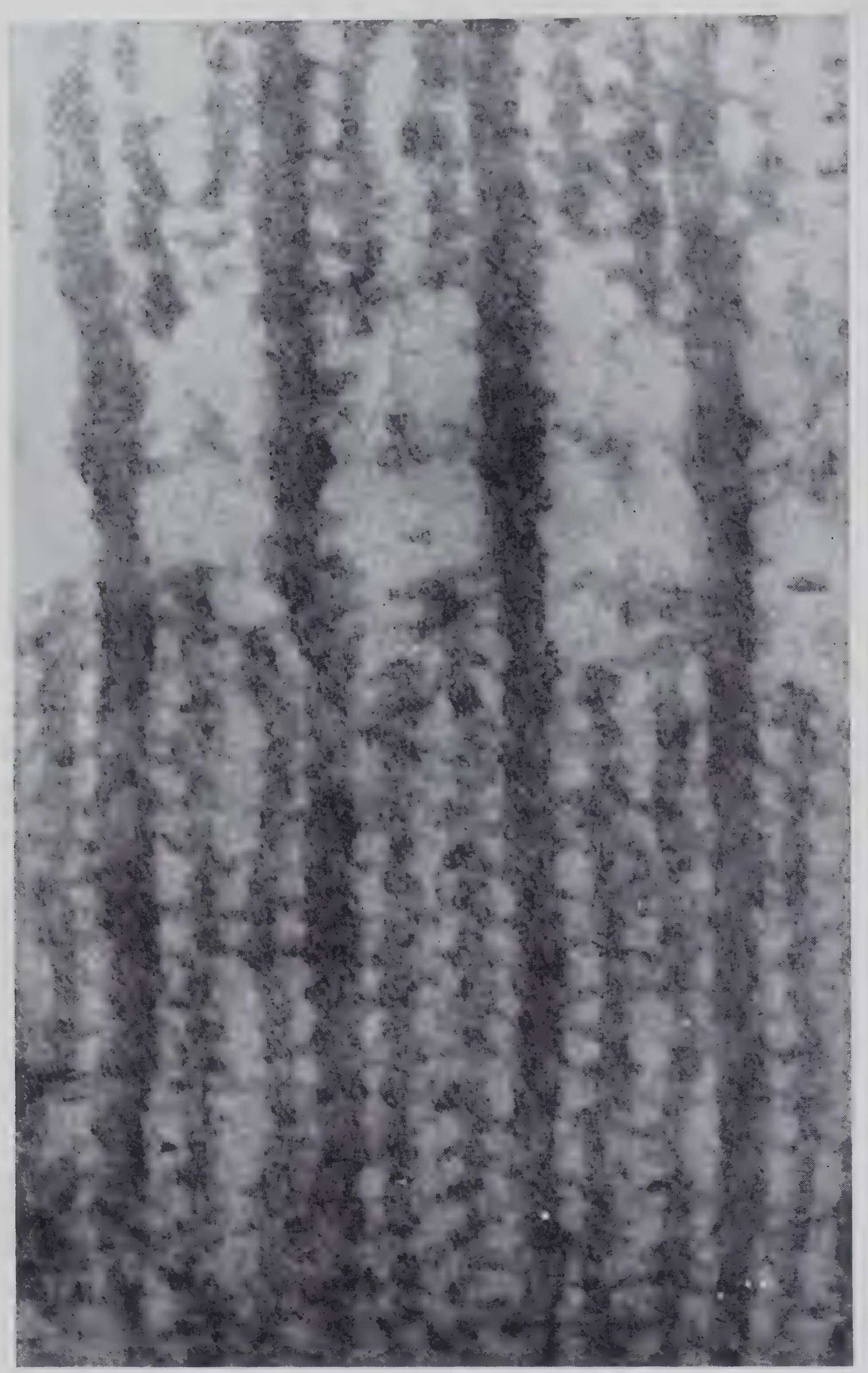


FIG. 14. Highly magnified electron micrograph through A-band of myofibril from rabbit psoas muscle, showing the system of cross connections between the large and small filaments. Magnification: ×444,000.

ment of the bridges is such that one of them occurs about every 400 A. between any given thick and thin filament. This dimension cannot be measured directly, for dimensions in very thin sections are in general greatly altered by flow of the plastic during sectioning. However, the number of such bridges in one half A-band can be counted (there are about 18) and their separation computed from the length of the A-band as measured in the light microscope. Each primary filament forms cross bridges to the six secondary filaments around it, and so there are six such bridges on each primary filament in each 400-A. length. It can be seen in the micrographs that these six bridges do not all occur in register, but seem to be spaced out evenly, probably at 60–70 A. intervals. The bridges do not attach to the secondary filaments (which have three such connections per 400 A.) in register either, and here too they seem to be spaced out evenly, this time at about 130 A. intervals. These arrangements must have a close relation to the size and the shape of the actin and myosin molecules and the way in which they are put together in the filaments.

One final observation concerning the cross bridges: they show small amounts of variation in their spacings, and small variations in their angle of tilt, but by and large they are roughly at right angles to the filaments, and their appearance is not different in stretched muscle, in the region of overlap, nor in muscle which has shortened. This shows quite clearly that any movement of the bridges during contraction must be a cyclic one.

3. *Light Microscope Observations of the Location of Muscle Proteins*

a. Historical. Ideas about the biochemical structure of striated muscle fibrils had fallen into a state of considerable confusion by 1950. It had earlier been pointed out that the birefringence of the A-bands could be readily accounted for if the "myosin" of the muscle was located in them [e.g., T. H. Huxley, 1880 (an amusing reference found by A. F. Huxley); von Muralt and Edsall, 1930; Weber, 1934]. However, it was subsequently discovered by Straub (1942) that the extracted protein previously referred to as "myosin" on the assumption that it consisted of one protein only was in reality a complex of two proteins—myosin and a new structural protein, actin; this complex was named actomyosin. And it had been demonstrated by A. Szent-Györgyi (1942) that threads of "myosin" (in fact actomyosin) would contract in solutions containing ATP. These findings, which showed that it was actin and myosin *together* which gave a contractile system, led to the idea that "actomyosin" must be present along the whole length of the muscle; for otherwise, how could shortenings of 70–80% be produced (as in toad sartorius muscle or in glycerinated rabbit muscle) when the A-band accounted for only 60% of the length of the sarcomere? Strength was added to this concept by the appearance, in the earlier electron micrographs, of seemingly continuous filaments, presumed to contain actomyosin, extending from one end of the sarcomere to the other.

This picture of the structure of muscle led to great difficulties. In the first place, one would expect the actomyosin filaments to show strong birefringence along their whole length; the low birefringence of the I-bands, in all media, made it necessary to presume that some other oriented material was present there which had a negative form and intrinsic birefringence of just the right magnitude to cancel out the positive form and intrinsic birefringence of the actomyosin for all values of the refractive index of the surrounding medium. It was claimed on several occasions that negatively birefringent material had been extracted from the muscle, leaving the muscle uniformly birefringent along its length, or that extraction of actomyosin left behind negatively birefringent segments (Dempsey *et al.*, 1946; Matoltsy and Gerendas, 1947, 1948; Gerendas and Matoltsy, 1948; Matoltsy, 1949; Aloisi *et al.*, 1952, 1954). These reports have not been confirmed either by Dr. Jean Hanson and the present author or by other workers, and it seems possible that the negative birefringence, seen in the muscle sometimes, might arise from an artifact in the wax-embedding procedure used; it has never been seen in unembedded tissue by us.

The concept that actomyosin filaments extended uniformly through both A-bands and I-bands also made it necessary to postulate the existence of an "A-substance," in order to account for the high density of the A-bands. This substance was supposed to migrate to the Z-lines and give rise to contraction bands when the muscle shortened.

b. Recent qualitative observations. The suggestion that myosin might be located in the A-bands was repeated by later authors (Amberson *et al.*, 1949, 1950; Ashley *et al.*, 1951; A. Szent-Györgyi, 1951), but the evidence was always of a rather indecisive character. In 1951, however, Hasselbach and Schneider showed that in the presence of 0.01 M pyrophosphate, myosin can be extracted selectively and completely from minced whole muscle (i.e., from broken muscle fibers) by solutions of high ionic strength. This paved the way for experiments to find out where the myosin was located in muscle. These were carried out by Hasselbach (1953) and by Jean Hanson and H. E. Huxley (1953). It was found that when minced muscle—the classical material—was examined histologically after the extraction of myosin from it, the dense material of the A-bands had been removed. The two latter authors were also able to perform the same type of experiment on isolated myofibrils, which could be examined throughout the extraction process in the phase contrast light microscope.

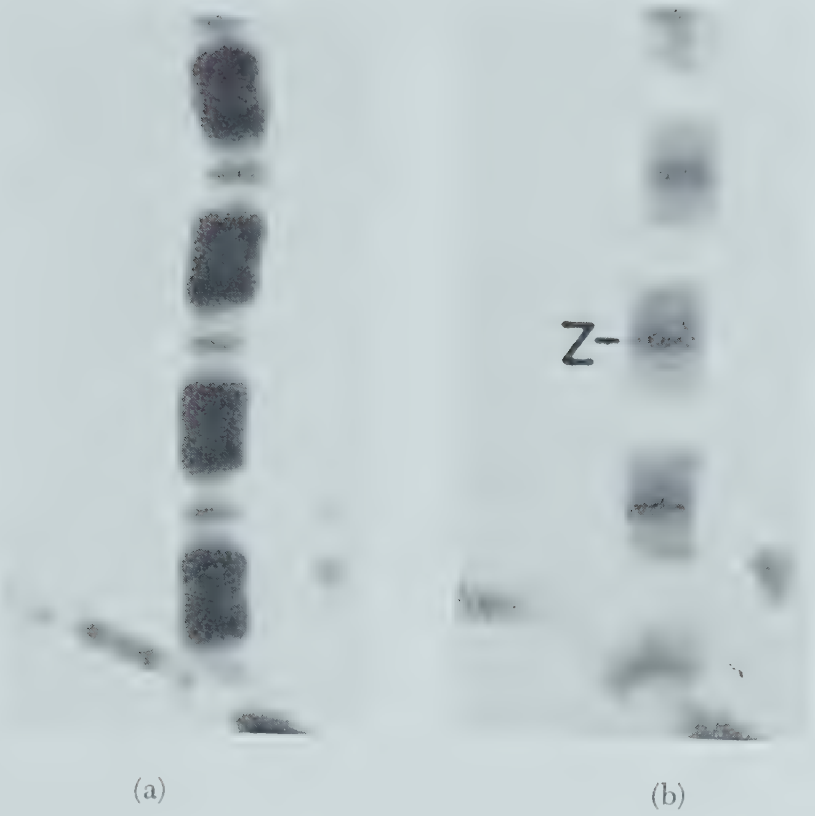


FIG. 15. Isolated myofibril from glycerinated rabbit psoas muscle in phase contrast illumination. a. Intact. b. After irrigation with 0.6 *M* KCl, 0.01 pyrophosphate, 10^{-3} M. $MgCl_2$ pH 6.5 to extract myosin. The extra dense material which makes up the A-band is removed, and one is left with the Z-lines and the secondary material extending from them up to the edges of the H-zones.

The removal of the extra material in the A-band, illustrated in Fig. 15, is a very striking and remarkable phenomenon indeed. The birefringence of the A-bands disappears at the same time, and "ghost" fibrils left behind have a low positive birefringence (H. E. Huxley, 1955). When the "ghost" fibrils are examined in the electron microscope, it can be seen that the array of thick filaments which formed the A-bands in the intact fibrils are no longer present; only thin filaments are visible now.

These ghost fibrils consist of the Z-lines and material (thin filaments) stretching from them to the edges of the former H-zone (the less dense region in the center of the A-band). If they are treated with 0.6 M KI, a procedure known to remove actin from muscle (A. G. Szent-Györgyi, 1951a, b), then a further extraction of material takes place, and one is left with the Z-lines alone (see Fig. 16) joined together by some "backbone" material of very low density which cannot be seen in the phase

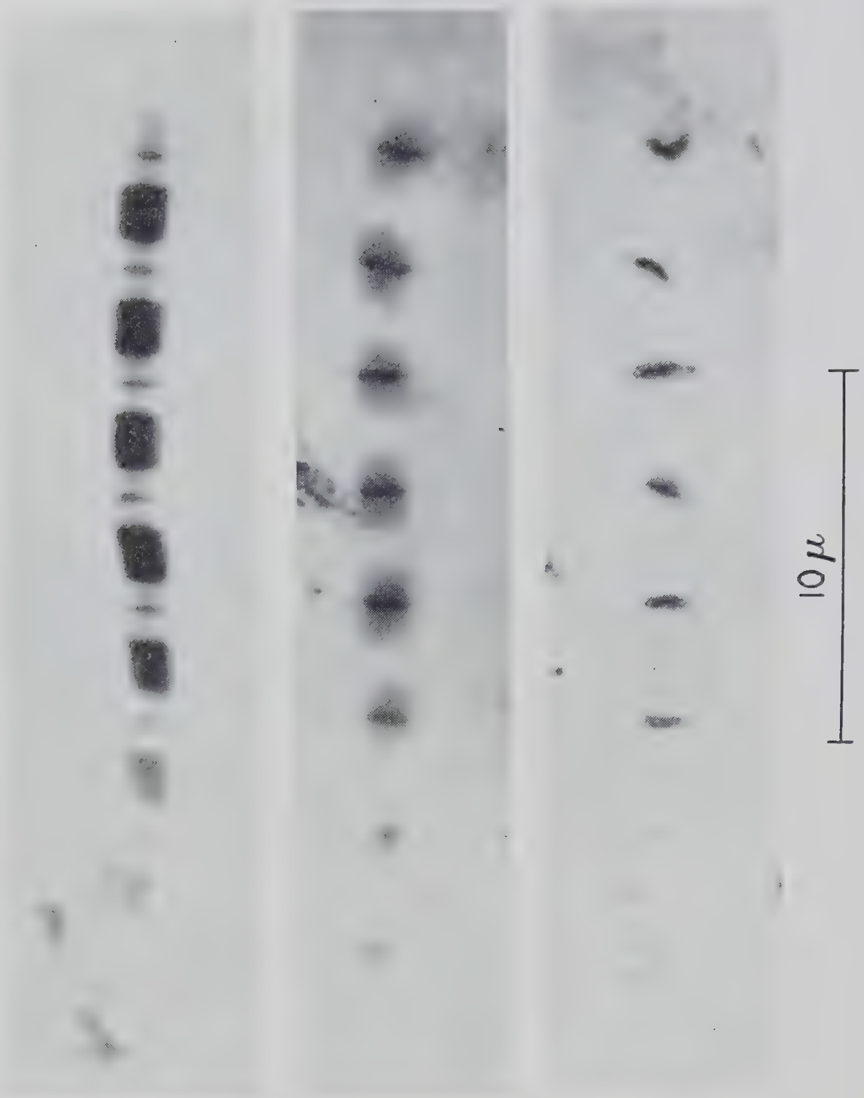


FIG. 16. Isolated myofibrils from glycerinated rabbit psoas muscle in phase contrast illumination. a. Intact. b. After myosin extraction. c. After irrigation with 0.6 M KI to remove actin. A large amount of the secondary material disappears when actin extraction takes place, leaving behind the Z-lines connected together by some residual backbone substance. (Hanson & Huxley, 1955.)

contrast microscope, but which preserves structural continuity between adjacent Z-lines.

These observations provide rather strong qualitative support for the idea that the myosin component of the muscle is contained in the hexagonal array of thick filaments which give the A-bands their high density and birefringence; and that the actin component is contained in the second array of filaments which extend from the Z-lines to the edges of the H-zone. The actin filaments interdigitate with the myosin filaments in the A-bands on either side of the H-zone, and it is in these regions that "actomyosin" can exist.

c. Quantitative measurements in the interference microscope. Recently,

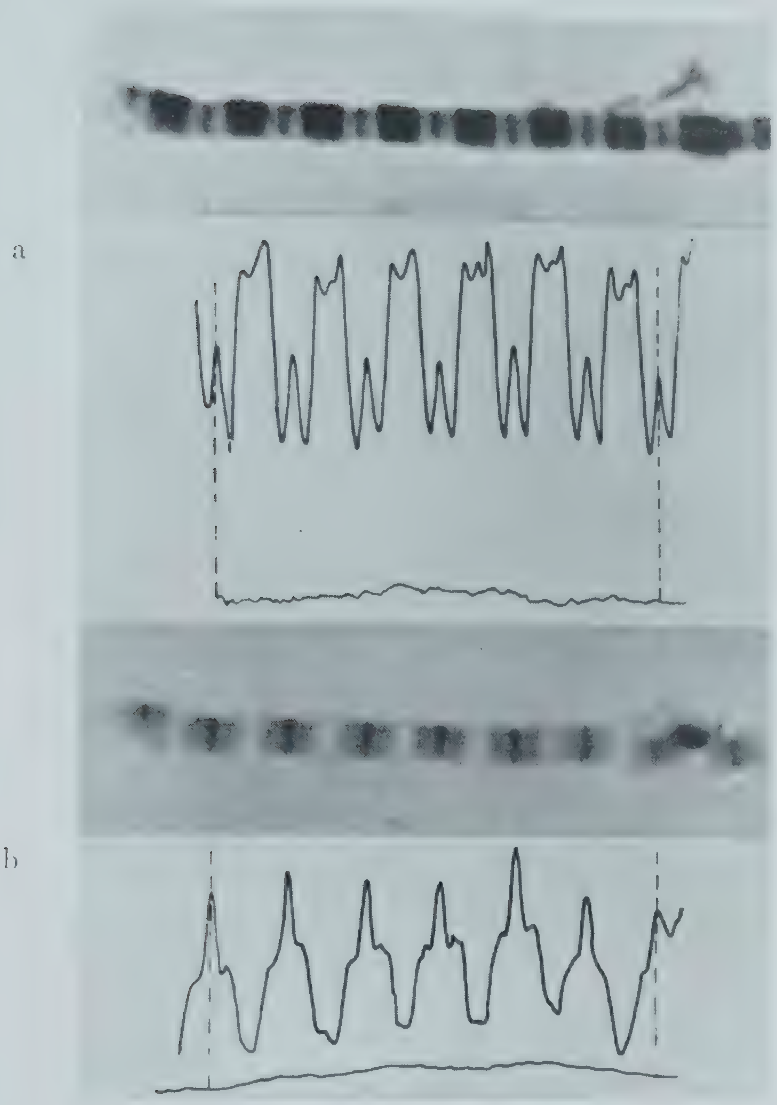


FIG. 17. Isolated myofibril photographed in interference microscope, and densitometer tracing along its length showing the distribution of protein between the different bands. a. Intact. b. After myosin extraction. The whole of the extra dense material in the A-bands is removed; a small decrease also takes place in the level of the I-band density. (Huxley & Hanson, 1957.) (By courtesy of *Biochim. et Biophys. Acta.*)

it has been possible to put these observations of the location of myosin on a quantitative basis (H. E. Huxley and Hanson, 1957; Hanson and Huxley, 1957). The interference microscope was used (Davies *et al.*, 1954; Davies and Deeley, 1956). This instrument permits measurement of the phase retardation of the light beam as it passes through any chosen part of the specimen. From such measurements it is possible to calculate the amount of protein present in the different bands of a muscle fibril; this can be done both before and after myosin extraction, so that the total amount of portein extracted, and the amounts from the different bands, can be determined (Fig. 17). These quantities can then be compared with large-scale biochemical analyses of the same material.

TABLE III

INTERFERENCE MICROSCOPE MEASUREMENTS ON STRIATED MUSCLE

	Quantity expressed as % of total myofibrillar protein[a] Method 1	Method 2	Method 3
Amount of A-substance	54.5 ± 2.0	55.3 ± 2.0	49.6 – 54.0
Amount of I-substance	36.4 ± 2.0	35.7 ± 2.0	36.5 – 37.9
Amount of extra material in Z-lines	6 ± 1	6 ± 1	—
Amount of material in H-zone gap after extraction	3.1 ± 0.3	(3.1 ± 0.3)	3.5 – 6.9
Amount of A-substance extracted	54.5 ± 2.0	—	—
Amount of I-substance extracted	10.0 ± 1.0	—	—
Total amount extracted	64.5 ± 3	61.5 ± 4	—

[a] The three methods represent different ways of analyzing the experimental results; see original paper for details (H. E. Huxley and Hanson, 1957).

TABLE IV

BIOCHEMICAL ANALYSES OF STRIATED MUSCLE[a]

	As % of total protein in fresh muscle			As % of total protein in glycerinated muscle			As % of total protein in washed fibrils		
Protein fraction	H-S	S-G	H-H	H-S	S-G	H-H	H-S	S-G	H-H
Soluble	28	(48)	34	—	42	28	—	—	—
Myosin	38	(26)	34	—	29	37	(53)	50	51
X-Protein[b]	—	(9)	7	—	10	8	—	18	11
Myosin + X-protein	—	(35)	41	—	39	45	—	68	62
Actin	14	—	—	—	—	—	(19.5)	—	—
Actin + residue	34	(17)	25	—	19	27	(47)	32	38

[a] Analyses by Hasselbach and Schneider (H-S) (1951), A. G. Szent-Györgyi *et al.* (S-G) (1955), and Hanson and Huxley (H-H) (1957). Figures in parentheses calculated by H. E. Huxley and Hanson (1957).

[b] Extra protein, extracted at the same time as myosin, but not precipitated at $\mu = 0.04$.

The results of these experiments can be stated very simply and are given in Table III. The extra material of the A-band, as measured in the interference microscope, amounts to 50–55% of the total protein in the myofibril. When myosin extraction is performed, all of this material, together with a further 10% of the total myofibrillar protein which is extracted from the I-material (i.e., the material stretching from the Z-lines to the H-zones), is removed. Biochemical analysis of the same process shows that a total of about 62.5% of the total myofibrillar protein is removed; 51% of the myofibrillar protein (i.e., about 80% of the extract) can then be precipitated under conditions which are classically used to precipitate myosin, while the remainder (~11%) remains in solution. These results show that at least four-fifths of all the myosin *must* be present in the A-bands, and they are in perfect agreement with the hypothesis that *all* the myosin is located there, as the A-substance, and as the model requires.

The results of these and of other analyses of muscle are summarized in Table IV. Soluble proteins, which no doubt include many of the enzymes involved in the glycolytic and oxidative processes, make up about 34% of the total dry weight of whole rabbit psoas muscle. The I-substance (excluding the extra material at the Z-lines which accounts for about 6% of the fibrillar protein) makes up about 36% of the protein in the myofibrils or about 25% of the dry weight of whole muscle. This is substantially more than the amount of actin which anybody has been able to extract from muscle; and so there is an indication that some other protein may also be present in the secondary filaments. We will take up later (Section III, A, 2, C and 3, B) the question of whether this may be the rather elusive tropomyosin.

4. *Changes in the Band Pattern of Muscle during Contraction and Stretch*

a. Historical. We have seen in the preceding sections that the pattern of striations arises directly from the way in which the contractile proteins of the muscle are organized into the two separate but overlapping sets of longitundinal filaments. It is therefore apparent that knowledge of the behavior of the pattern of striations during active or passive changes in the length of the muscle will give rather direct information about the behavior of the actin and myosin filaments during these processes. It is a very fortunate state of affairs that what we can see in the light microscope should be so straightforward to interpret in terms of what is happening at the level of the molecular filaments. But the light microscope information has proved more difficult to gain than might at first be supposed. The observations are beset by optical artifacts which arise because of the large thickness of the fibers in comparison with the lengths of the bands: because of the different refractive indexes of the different bands: because of alterations in band lengths which tend to take place during any fixing, embedding, and sectioning, whether for light or electron microscopy: and because of the high speed at which the changes take place during normal contraction. These factors account for a good deal of the conflict which has existed between different observations of band changes. The history of these conflicts, and the reasons for them, have been described by Huxley and Hanson (1954) and particularly engagingly by A. F. Huxley (1957), and need not be repeated here.

b. Recent measurements. It is clear now that there are only two reliable methods at present available for measuring accurately the band lengths in striated muscle. These are either (1) to use whole fibers immersed in a medium whose refractive index matches the average refractive index of the whole fiber, eliminating the "cylindrical-shape artifact" (A. F. Huxley, 1957) and to examine the striations in a phase contrast or interference microscope with a sufficiently small depth of focus to show only a thin optical section of the fiber, or (2) to use isolated myofibrils, so that the thickness of the specimen ($\sim 1\,\mu$ or less) introduces little error into the measurement of band length, which can be made in the phase contrast light microscope. Method (1) has been used by A. F. Huxley and R. Niedergerke (1954) and method (2) by Dr. Jean Hanson and the present author (Huxley and Hanson, 1954).

The former authors examined living frog fibers during passive stretch, and in isometric and isotonic contractions, both in twitches and in tetani. Hanson and Huxley used isolated rabbit myofibrils, which could be made to contract by irrigating them with solutions of ATP, and whose length could be controlled by a form of micromanipulation. The use of isolated fibrils has the disadvantage that it is one step removed from the living muscle, but it has the advantage that the band changes, in particular those occurring in the H-zone, can be seen more clearly than in whole fibers; moreover, myosin extraction can be performed on the fibrils at any chosen stage in the process, making interpretation of the observations much more straightforward.

The results of the measurements are very simple. Both sets of authors found that over a wide range of muscle lengths the length of the A-band does not change perceptibly, either during stretch or during isotonic or isometric contraction. Changes in the length of the sarcomere are accounted for by changes in the length of the I-bands alone. Figure 18 illustrates the results obtained on myofibrils.

When the muscle has contracted down to about 60% of its resting length (at which the lengths of the A- and I-bands were $1.5\,\mu$ and $0.8\,\mu$

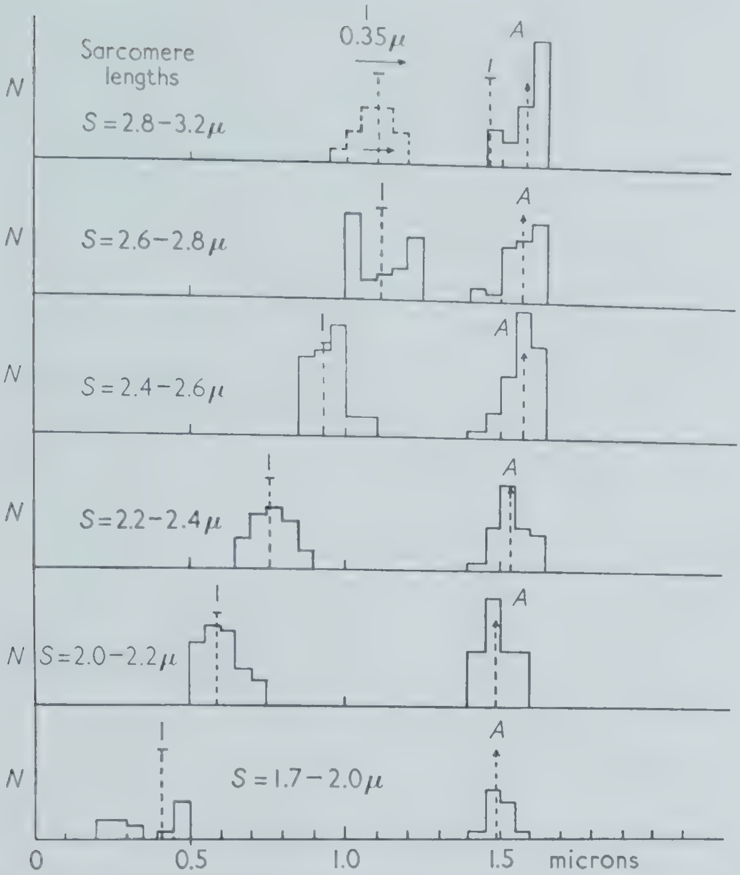


FIG. 18. Histogram showing results of measurements of band lengths on isolated myofibrils from glycerinated psoas muscle at different sarcomere lengths. Fibrils with sarcomere lengths of 2.4 μ or more were obtained from fibers held stretched during glycerination; those with sarcomere lengths of less than 2.2 μ had been caused to contract by irrigation with suitable amounts of ATP.

respectively), the I-bands disappear and the A-bands come into contact with the adjacent Z-lines. If contraction proceeds beyond this point (which under physiological conditions it usually does not), then contraction bands form around the Z-lines. The constancy of the length of the A-band over the working range shows that the thick filaments which make up the A-band must remain at constant length during both stretch and contraction, down to the point at which their ends, for steric reasons, have to crumple up.

The behavior of the secondary filaments can be deduced from the other changes which take place in the band pattern. In isolated myofibrils, the H-zones are seen to be much wider in stretched material than in fibrils at rest length (see Fig. 19). During shortening, the H-zones become narrower and disappear at about 85% R.L., being replaced on further shortening by a dense line. If myosin extraction is performed on fibrils at the different stages of shortening, it is found that the different lengths of the H-zone are duplicated by the different lengths of the gap between

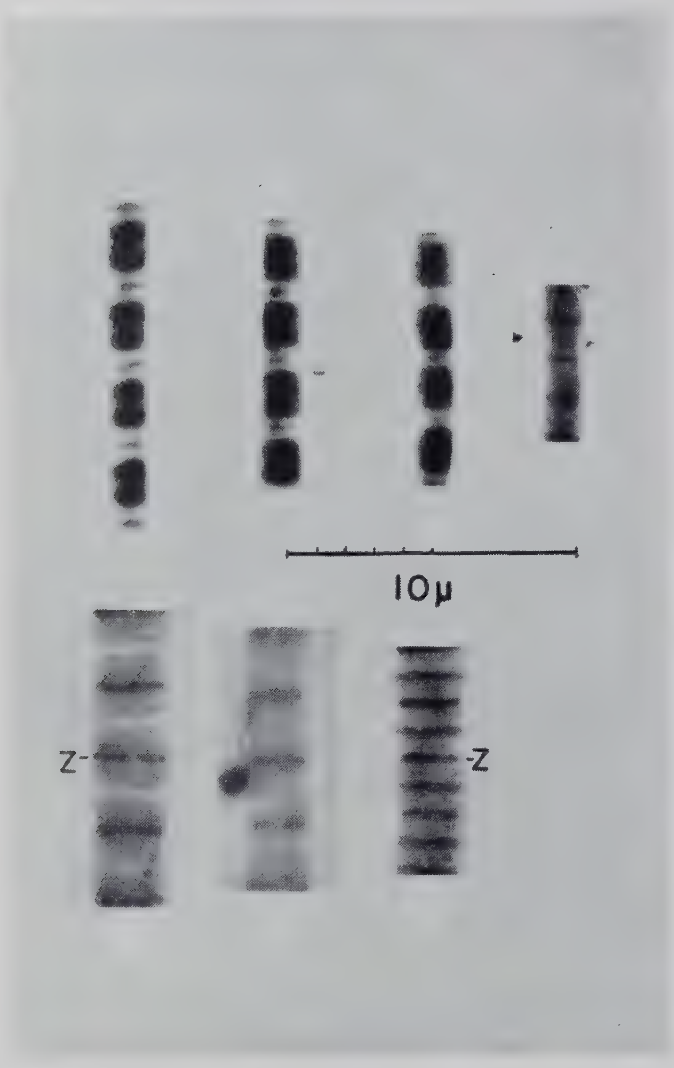


FIG. 19. a. Isolated myofibril from glycerinated psoas muscle made to contract in a number of stages by irrigation with a succession of small amounts of ATP. Note the changes in the H-zone which occur while the I-bands are shortening and the A-bands are at constant length. The I-bands eventually disappear and are replaced by contraction bands where adjacent A-bands come into contact at the Z-lines. b. Fibrils, at the same sarcomere lengths as in the contraction series, from which myosin has been extracted, showing more clearly the closing up of the H-zone, and its replacement by a dark line, as the sarcomere length is reduced. (Hanson & Huxley, 1954.)

the ends of the secondary material, and that in fibrils where the H-zone has been replaced by a dark line, this line remains after extraction. Myofibrils in these various conditions are shown in Fig. 19. Down to the point where this line forms, that is, from about 140% R.L. (the limit of stretch) to 85% R.L., the distance from the Z-lines to the edge of the H-zone (or to the edge of the gap after myosin extraction) remains constant. It is apparent from these observations that the secondary filaments, too, do not change their length perceptibly over a considerable range of muscle lengths.

c. Other observations on band lengths. Measurements on isolated myofibrils in the phase contrast light microscope have also recently been reported by Villafranca (1957). The results are very similar to those which have been described above; the A-band remains apparently constant in length to within ±0.075 μ (i.e., any changes in length are less than the resolving power of the light microscope), over the range of sarcomere lengths used, 2.3–3.3 μ. The I-bands were, of course, not constant in length, and the H-zones, too, were found to be much longer in stretched muscle, although the observed differences in the latter case were somewhat smaller than would have been anticipated; however, this measurement is a difficult one to make accurately, for the density difference at the boundaries is rather small.

Carlsen and Knappeis (1955) and Knappeis and Carlsen (1956) have reported measurements on band lengths in intact fibers in papers which continue the earlier work of Buchtal *et al.* (1936). The results obtained by these workers, who found considerable changes in A-band lengths, are not in agreement with the ones we have described above, which were obtained under conditions where optical artifacts were minimized. The optical artifacts which may have been present in the former measurements have recently been discussed by A. F. Huxley (1957).

It may also be of some interest to compare low-power electron micrographs of rest length and of stretched muscle. In order to compare band lengths it is necessary to cut sections with the microtome knife parallel to the fiber axis, so that any compressive flow of the plastic will not affect the measurements. If this is done, and if the muscle is held at a fixed length during fixation, dehydration, and embedding, it will be seen that the A-band length is virtually constant at the different sarcomere lengths (Fig. 20, A). Moreover, the constancy in the distance from the Z-lines to the edges of the H-zone may be seen in the same pictures (Fig. 20, B). These observations cannot of course be accepted as independent evidence for these characteristics of the band pattern, for one cannot establish in this type of experiment that no changes have taken place in the relative A- and I-band lengths during fixation. They do, however, provide perhaps a clearer visualization of the changes that have been seen in the light microscope.

Sjöstrand and Andersson-Cedergren (1957) have reported observations of an apparent decrease of both A- and I-band lengths at sarcomere lengths below rest length. These changes were not associated with changes in the *pattern* of bands such as we have been discussing; and the present author has observed such effects only in specimens which were incorrectly oriented during sectioning, so that compressive flow reduced the apparent band lengths.

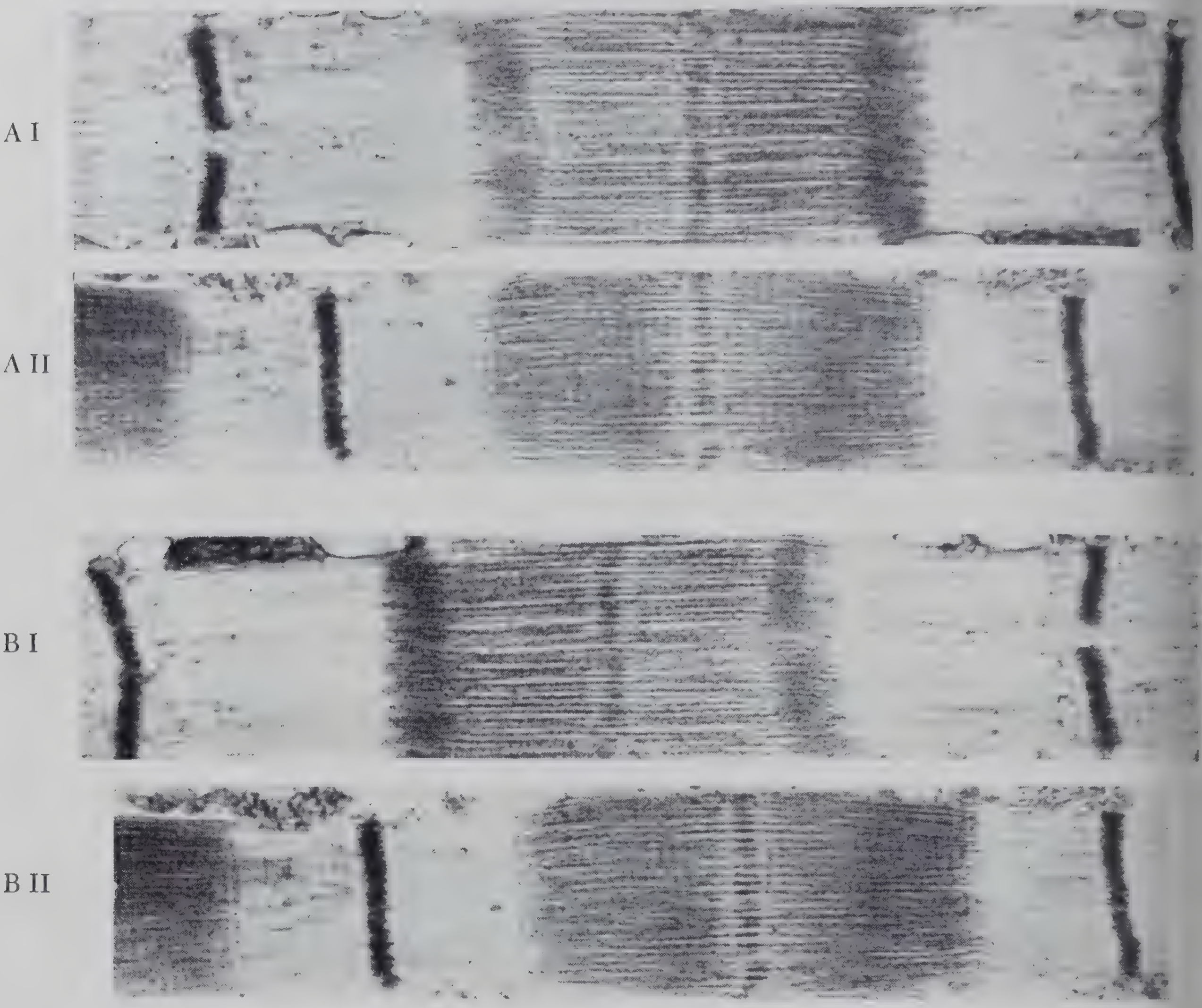


Fig. 20. The changes in band pattern at different muscle lengths as seen in the electron microscope (thick sections; oriented for sectioning so as not to foreshorten band lengths. A, I. Stretched muscle showing long I-bands and H-zone. A, II. Rest-length muscle, showing decrease in length of I-bands and H-zone, and constancy of length of A-band. B, I and II. Same pair of preparations showing constancy of distance between Z-lines and edge of H-zone. Magnification: ×30,000.

d. Conclusions. Apart from the dissenting opinions we have mentioned, it seems reasonably clear that changes in the length of the muscle, whether active or passive, over a substantial range of lengths, are the result of a process in which the two arrays of filaments slide past each other. It is unlikely that muscle would contract by one process over one part of its range and by a different process over the other, and it can be concluded that the sliding process is the fundamental one over the whole range; any folding up of actin or myosin filaments most probably takes place as a result of shortening rather than as its cause. Thus the essential mechanism of contraction must be one which causes the two sets of filaments to slide past each other.

C. Other Considerations Which Support the Interdigitating Filament Model

So far, the structural evidence alone has been considered. This evidence is strong and self-consistent, and it is not unsupported. Many other lines of evidence, some of an extremely important character, also have a strong bearing on the model we are discussing, and this will be an appropriate place at which to introduce them.

1. Behavior of Fiber and Thread Models

The biochemical behavior of a living muscle fiber is, like that of any other intact cell, extremely difficult to study directly. The presence of the cell membrane impedes the free diffusion of substances whose effect or whose production we wish to investigate. The phenomenon of excitation, of contraction, and of relaxation, are all present together, and in the interpretation of any particular experimental result it is not easy to disentangle the contribution of each. Moreover, the range of conditions of pH and of ionic strength over which the system may be studied in a functional state is quite small. The conventional procedure of disrupting the tissue and studying cell-free extracts is satisfactory up to a point; but it is then no longer possible to correlate the chemical reactions observed with what, after all, is the function of the tissue—to shorten and to exert a tension.

a. Fiber Models. It was found by A. Szent-Györgyi (1949) that if small bundles of rabbit psoas muscle fibers were kept at –20° C. in a 50% glycerol-water mixture, they would contract as soon as they were placed in a suitable salt solution containing $5 \times 10^{-3} M$ ATP, even after months or years of storage. These so-called "glycerinated" muscle fibers no longer possess an electrically excitable membrane, their internal supplies of ATP and creatine phosphate (CP) have been leached out, and their metabolic systems have been disrupted; but their contractile machinery seems to be virtually intact. They provide almost the ideal experimental material. The fibers can be placed in whatever medium we choose and we can study the effect of the medium on the contractile elements alone. The disruption of the cell membrane allows diffusion to take place quite readily, and if it is desirable to reduce diffusion effects through the muscle substance itself still further, the glycerinated fibers may be broken into isolated myofibrils, in which the structural integrity of the contractile elements is preserved. It has not so far been possible to measure tension production by isolated fibrils, but the changes in their length and band pattern can be readily observed in the light microscope.

Glycerinated fibers will exert a tension at least as great as that of intact muscle, and they imitate its behavior quite closely in many other ways too (see reviews by Weber and Portzehl, 1952, 1954). They shorten more slowly than living muscle fibers, but this is due, in part at least, to the supply of ATP to the inside of whole fibers being diminished by diffusion effects; isolated fibrils contract in $5 \times 10^{-3} M$ ATP in a fraction of a second (Hanson and Huxley, unpublished observation). Glycerinated fibers exert their maximum tension around rest length and lower tensions at shorter lengths. They exhibit the "quick-release" phenomenon, i.e., if they are allowed to shorten rather quickly, the tension they exert will drop to zero and then redevelop at the shorter length. The efficiency with which they transform chemical energy into mechanical work may be as high as 13% (W. Falk quoted by Weber and Portzehl, 1954). Hill's estimate (1949c) for the highest efficiency of living frog muscle, expressed in comparable terms (i.e., efficiency of utilization of immediate energy sources), was about 40%. The molecular structure of the contractile elements, as studied by X-ray diffraction or electron microscopy (H. E. Huxley, 1953a, b) appears to have been preserved extremely well by the glycerol treatment. All these observations give us confidence that the chemical events and their physical consequences, which can be studied in a precise and controlled way in this system, bear a very close relation to those taking place in the living muscle.

b. Thread models. Another type of model system, to which we have already referred (Section I, E), is the actomyosin thread. This can be prepared from a mixture of the proteins actin and myosin in solution in $0.6\,M$ potassium chloride. If such a solution is squirted as a fine jet into distilled water from a moving pipette, the salt ions will diffuse outward from the "thread" of protein solution much faster than the protein molecules themselves. Actomyosin precipitates when the ionic strength falls below $0.1\text{–}0.2\,\mu$, and so a fine thread of precipitated actomyosin is formed, in which the protein is oriented to some extent by its flow through the fine pipette. The thread can be further oriented and concentrated by suitable techniques (Portzehl, 1951), when it will contract anisodimensionally in ATP and will exert tensions of a few hundred grams per square centimeter. This system will also imitate, in a somewhat inferior fashion, many of the other properties of glycerinated fibers and of whole muscle. It seems to contain the essentials of a contractile system, put together in an inexact, and perhaps incomplete, way.

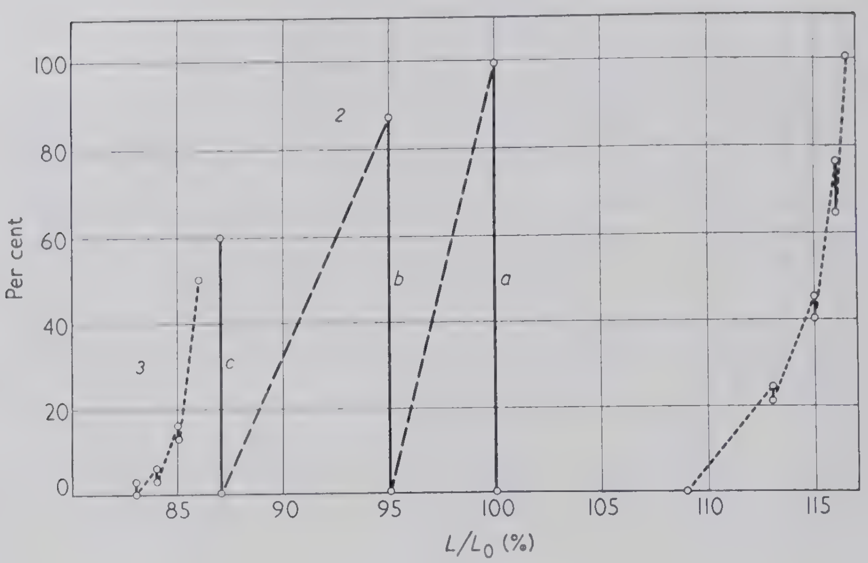
c. Principal results with fiber and thread models. Experiments on these models have led to the development of very simple and elegant concepts concerning the part played by ATP in muscular contraction and relaxation (A. Szent-Györgyi, 1951; Weber and Portzehl, 1952, 1954). These were originally formulated in chemical rather than structural terms, but they have a very immediate relevance to the structural model. It was these concepts, taken together with the low-angle X-ray evidence, which originally suggested that actin and myosin were located in two separate sets of filaments, and which, combined with later structural evidence, led to the interdigitating filament model and the sliding filament hypothesis.

The results in question can best be described with the aid of diagrams (taken from A. Weber, 1951; H. H. Weber, 1952; Weber and Portzehl, 1952, 1954), showing the course of tension development by glycerinated muscle fibers under various conditions. The fiber first develops tension on being immersed in a medium containing ATP. If it is allowed to shorten slightly, the tension falls during the release and then recovers again (Fig. 21, A) as the fiber redevelops tension at the new length. If the ATP is washed out while the fiber is held at a fixed length, the tension remains high, but if the fiber is then allowed to shorten slightly, the tension falls to zero and does not recover again. It seems that the contractile structure "sets" at a fixed length when the ATP is removed, and that the continuing tension is due merely to stretched series elastic elements.

In the next experiment (Fig. 21, B), the fiber is allowed to develop tension in ATP, and then, as before, the ATP is removed by washing; the tension remains high. The fiber is then bathed in a medium containing $2.5 \times 10^{-2} M$ sodium pyrophosphate, when the tension falls to a low value. If the fiber has been allowed to shorten in the ATP, it may now be re-extended by a small force. If ATP solution is again substituted, tension redevelops. Sodium pyrophosphate is not enzymatically split by myosin, but, like ATP, it possesses the property of being able to dissociate actomyosin. Other agents, such as triphosphate or ADP, which can dissociate actomyosin but are not themselves split, will cause fiber models to relax.

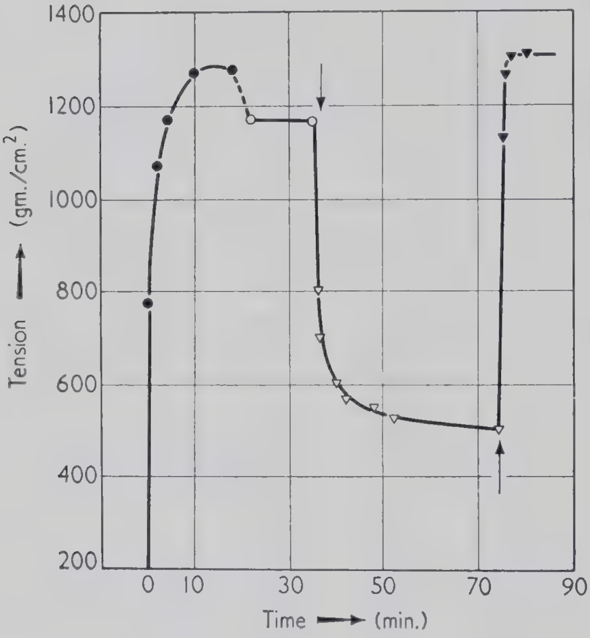
In another type of experiment (Fig. 21, C), the fiber is allowed to develop tension in ATP, and then Salyrgan is added to the bathing medium; this substance inhibits myosin ATPase. When this is done, a large decrease in tension occurs. In a similar type of experiment, an ATP solution which has produced tension is replaced by a solution containing ATP plus a "relaxing" factor which can be extracted from fresh muscle (Marsh, 1951, 1952) and which causes a partial inhibition of ATPase activity; when this is done, relaxation is again produced (Bendall, 1952; Hasselbach, 1952; quoted by Weber and Portzehl, 1954; Portzehl, 1957). It is also found that many other substances which inhibit ATPase activity cause fiber models to relax in ATP (Weber and Portzehl, 1952, 1954).

These experiments indicate that ATP breakdown is necessary for contraction; and that the presence of ATP under conditions where ATP breakdown does not take place leads to relaxation. Other agents which

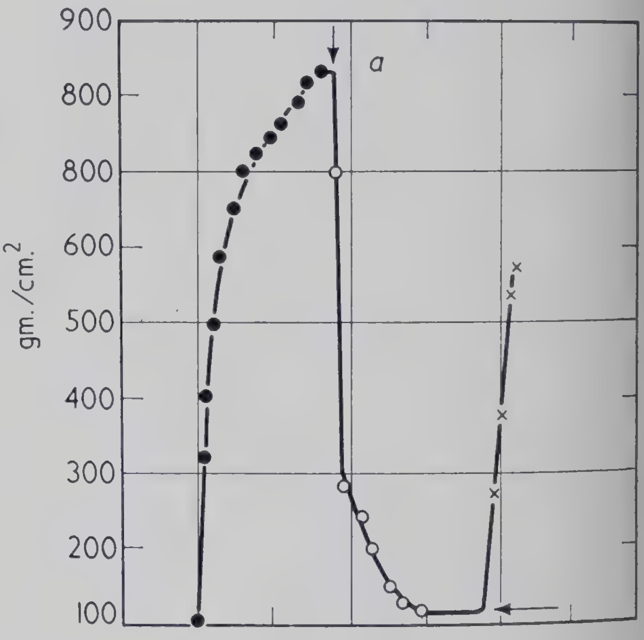


A

FIG. 21. Behavior of glycerinated psoas fibers in ATP. A. Redevelopment of tension in the presence of ATP, and elastic aftereffect in the absence of ATP, following release. *Curve 1:* stretched fiber released in absence of ATP; *curve 2***:** fiber released in presence of ATP; *curve 3:* fiber after washing out ATP. (A. Weber, 1951.) (By courtesy of *Biochimica et Biophysica Acta.*)



B



C

FIG. 21. B. "Relaxation" of glycerinated fibers produced by pyrophosphate; ●-●-●-●, ATP contraction; ––––, ATP washed out; o–o, without ATP; ↓ 0.025 *M* pyrophosphate; ↑ 3.10^{-3} *M* ATP anew. (H. H. Weber and Portzehl, 1954).

FIG. 21. C. "Relaxation produced by Salyrgan, reversed by cysteine; ●-●-●-●, ATP contraction; ↓, 1.10^{-4} *M* Salyrgan; ←, 2.10^{-4} *M* cysteine. (H. H. Weber & Portzehl, 1954.) (Courtesy of Pergamon Press.)

dissociate actomyosin also produce relaxation. In the absence of ATP or these other dissociating agents, the fibers "set" at a fixed length.

a. Conclusions. These biochemical concepts have a straightforward interpretation in the interdigitating filament model. The cross bridges in the model represent sites of ATPase activity and of actin-myosin combination. When ATP splitting takes place, the cross bridges act as structural agents which in some way cause the actin filaments to move, or to tend to move, past the myosin filaments. This process continues as long as ATP splitting is taking place, the bridges going through a cycle of changes each time an ATP molecule is captured and split; if the ATP is washed out of the muscle, the two sets of filaments become attached together in whatever position they happened to be, and the muscle "sets" at a fixed length. If ATP splitting is stopped while ATP is still present, then that ATP prevents the filaments from sticking together at the cross bridges; for these are sites of actomyosin formation, and ATP can keep actomyosin dissociated as long as it is itself not split. Thus the muscle relaxes. Other dissociating agents act in a similar way.

In living muscle, ATP is present the whole time. In the resting state, ATPase activity is low and the actin and myosin filaments are dissociated; as has been pointed out to me by Dr. Andrew G. Szent-Györgyi, it is not quite clear which is cause and which is effect in this case. This relaxed condition is associated with the presence of the relaxing factor. When the muscle is stimulated, the effect of the relaxing factor is inhibited in some way, ATP splitting commences, and contraction occurs; when the contraction is over, the relaxing factor again exerts its influence, ATPase activity ceases, the cross bridges between the filaments are disconnected, and the muscle returns to its resting, relaxed state.

2. *Behavior of Muscle in Rigor and at the Start of Contraction.*

It is a commonplace observation that when a muscle dies, it becomes rigid and inextensible. It may contract slightly as it passes into this state of rigor, but any shortening can be prevented by a relatively small force (Sandow and Schneyer, 1955). The state of rigor is characterized by the loss of ATP by the muscle (Erdos, 1943). This loss of ATP, as in the experiment where previously added ATP is washed out of a glycerinated fiber, would lead to the formation of fixed links between the actin and myosin filaments. We can see easily from the sliding filament model why this should cause rigor; the myosin and actin filaments must be relatively inextensible structures themselves (in order to exert tension during contraction), and linking them together and inhibiting the sliding movement between them must make the muscle itself inextensible.

A similar effect may also occur during contraction; a muscle becomes less extensible very soon after it is stimulated (Hill, 1949c), even before it has commenced to show external shortening. If cross links begin to form as a consequence (or as a cause) of ATP splitting, then, if it is that reaction which produces contraction, we would expect a high resistance to stretch to develop very early in the contraction cycle of the muscle.

These two particular considerations provided a good deal of initial support for the idea that actin and myosin were present in separate filaments which were cross-linked together only during contraction or when the muscle was in rigor (H. E. Huxley, 1952, 1953a).

3. *The Similarity in the Behavior of Muscles over a Wide Range of Lengths*

Although the maximum tension which a striated muscle can exert varies with the length at which the muscle is held, it is a rather striking feature of muscles that they can function as they do over such a very wide range of lengths, which may extend from 20 or 30% of rest length to 140% R.L. It has been shown by Abbott and Wilkie (1953) that essentially the same force-velocity equation describes the behavior of active striated muscle over a wide range of lengths, and Hill (1949f) has shown that over a considerable range of lengths (down to 50% R.L. using toad sartorius) the delay in the rise of tension after stimulation varies very little either.

These features follow quite naturally from the sliding filament model; the interaction at the cross bridges can produce tension, produce it in the same way, and produce it immediately, at all muscle lengths. If the filaments resist compression to a lower extent than they resist stretch (as one might expect long polymers to do), then the sarcomeres can continue shortening below the length of either type of filament; in extreme shortening, the central region of the thick filaments will still be under extensive stresses, while part of the tension generated in that region will be used to compress the ends of those filaments by pulling in the Z-lines against them.

The ability of a striated muscle to shorten below 60% of its resting length has no obvious advantage; in general, it will never be able to do so *in vivo*, and in fact there is evidence (Ramsey and Street, 1940) that muscle fibers are damaged if they do so *in vitro*. The property is essentially an accidental consequence of the sliding filament system. However, if the muscle shortened by some transformation of the kind in which a number of contractile units arranged in series went over from a "long" to a "short" form, then it would be most uneconomic to construct it so that it *could* operate over a 7:1 range of lengths; in a normal contraction, most of the contractile units would never operate.

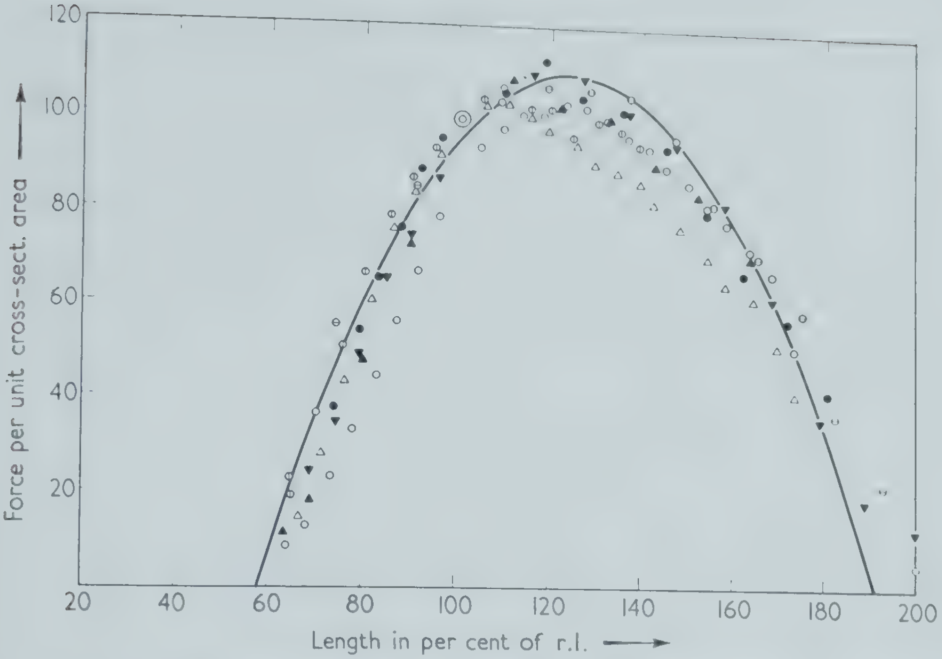


FIG. 22. Length-tension diagram, obtained from single muscle fibers showing decrease in tension development beyond rest length. Five independent sets of data by Ramsey and Street (1940).

Again, in the second type of model, a greater number of the contractile elements in a shortened relaxed muscle would, in the interval between stimulation and tension development, have either to shorten down if they were "lying slack" or else become locked in the shortened position if they were behaving plastically. The constant delay observed in practice would be very surprising if this model were correct, whereas it follows quite naturally from the sliding filament model.

4. *Shape of the Length-Tension Diagram beyond Rest Length*

Beyond rest length, the tension (above resting tension) that can be generated by a muscle when it becomes active falls off almost linearly with muscle length (see Fig. 22). It has been pointed out by A. F. Huxley and Niedergerke (1954) that this phenomenon provides strong support for the interdigitating filament model. The extent of overlap of the two sets of filaments falls off linearly with increasing muscle length; if the number of cross linkages formed does the same, and if tensions generated at the cross linkages add up in parallel, then the maximum tension should also decrease linearly beyond rest length.

This argument would be more satisfactory if the decrease in maximum tension at lengths shorter than rest length could be explained. Possibly the increase in fiber diameter which takes place (as the muscle contracts at constant value) allows the muscle filaments to move farther apart and so alters the spatial situation at the cross bridges.

5. *Quantitative Considerations*

As with any mechanism, it is important to see whether the system is practicable from a quantitative point of view. If a muscle can exert a maximum tension of 1.5 kg./cm.^{2}, if its heat of shortening is 350 gm.cm./cm./cm.^{2}, and if the maximum velocity of shortening is 10 muscle lengths per second, then the rate of energy release (excluding maintenance heat) by 1 cc. of muscle will vary from 3500 gm.cm. per second at zero load, to 1750 gm.cm. per second at half-maximum load, when the shortening velocity will be about 1.6 muscle lengths per second. If this energy is produced by the breakdown of an energy-rich compound which liberates 10,000 calories per mole, then the number of molecules of it broken down per second in 1 cc. of muscle will vary from 5×10^{18} to 2.5×10^{18}. From the geometry of the structure as we have described it, it is easy to show that the same volume of muscle contains about 5×10^{16} cross bridges. Thus if the cross bridges correspond to sites of enzymatic activity where energy liberation takes place, then each site breaks down 50 molecules of substrate per second at half-maximum load and 100 molecules per second at zero external load. Thus, in the former case, where the two sets of filaments are moving past each other at a speed of 18,400 A. per second (half sarcomere length is 1.15 μ) one molecule of substrate is broken down at each site, on the average, for each 300–400 A. of this movement.

These figures are quite sensible ones, and we may conclude that the general scheme is satisfactory in quantitative terms.

6. *Special Consequences of the Interdigitating Filament Model*

There are two further features of the model which distinguish it rather sharply from other types of mechanism (H. E. Huxley, 1956; A. F. Huxley, 1957). In this system, the length of the muscle is not directly related to the configuration state of the contractile sites. *Changes* in muscle length are produced by *changes* in configuration, but the configurational changes are repetitive. A good deal of discussion has gone on during recent years as to whether certain chemical events (for instance, the binding, and the dephosphorylation, of ATP) occur during contraction or during relaxation. These questions still have a meaning, but they must now be asked in terms of the individual cycles at each link, and the events taking place there when tension is being generated, or when the link is being transferred to the next site on the actin filament, rather than in terms of the contraction and relaxation of the muscle as a whole.

As a result of the repetitive operation of the links, each enzyme site can function a number of times during each contraction of the muscle. This would seem to be a more powerful system than one in which each of the sites, associated with the shortening of contractile units arranged in series, could operate only once per contraction. Indeed, such cyclic behavior is more or less implicit in the definition of the catalytic action of an enzyme. Furthermore, the possibility of a site operating a variable number of times in a constant distance of shortening would allow the muscle to release varying amounts of energy according to the loading conditions (cf. Needham, 1950).

D. Conclusion

Very strong evidence, both direct and indirect, now seems to exist for the general validity of the sliding filament structural model of striated muscle. The structure itself seems correct, as do also the changes in structure which the model predicts at different lengths. The evidence is less satisfactory however when we come to enquire exactly how those changes have been produced; the process may be more complicated than the word "sliding" suggests.

We will now return to a more detailed examination of biochemical, mechanical, and thermal observations on muscle; wherever possible, we will try to relate these to the structural concepts that have been formed.

III. The Muscle Proteins, Their Structure and Reactions and Their Behavior in Muscle

The two most direct questions which one might now ask are the following: (1) How exactly is this particular structure built up from the molecules of protein which we believe form the different parts of it? (2) Do any of the known properties of these proteins help us to understand how the sliding process could be brought about? We will keep these questions in mind while we discuss the physical and chemical properties of the proteins concerned.

A. Size, Shape, and Quantity of the Structural Proteins of Muscle (see Table V)

1. Myosin

a. Size and shape. Myosin is an elongated molecule with a high molecular weight. Estimates of the molecular weight have varied, but present figures suggest a minimum value of 400,000–500,000 rather than the earlier reports of about 800,000. Laki and Carroll (1955) find that freshly prepared myosin which has not been exposed to a temperature of more than 10° C. has diffusion and sedimentation constants given by $D_{20}{}^{W} = 1.0 \times 10^{-7}$ and $S_{20}{}^{W} = 6.15 \times 10^{-13}$; a specific volume of 0.728 then leads to a molecular weight of about 500,000. If the myosin is left at room temperature for about 2 hours the sedimentation constant increases

TABLE V

SIZE, SHAPE, AND QUANTITY OF STRUCTURAL PROTEIN OF MUSCLE

Protein[a]	Molecular weight	Length	Width	$S^{W}_{20} \times 10^{13}$	$D^{W}_{20} \times 10^{-7}$	% of dry weight of whole muscle	% of dry weight of isolated and washed myofibrils
Myosin[1–6]	400,000 –800,000	1500 A. –2300 A.	~25 A.	6.15–7.1	0.9–1.05	34–38[7, 8]	50–55[8]
F-Actin[9]	?	?	~50 A.	~50	—	~14[7]	19.5
G-Actin[10, 11] (monomer)	57,000 –70,000	~290 A.	~24 A.	3–4[9, 12]	—	—	—
G-Actin[11] (dimer)	~140,000	~580 A.	~24 A.		—	—	—
Tropomyosin[13]	53,000	340 A.	11–13 A.	—	—	10–12	15–18
Heavy meromyosin[14]	232,000	435 A.	29 A.	6.96	2.91	15.5–17	22.5–25
Light meromyosin[14]	96,000	400–550 A.	16.6 A.	2.86	2.87	18.5–21	27.5–30

[a] References identified by superscript numbers: [1] Portzehl (1950). [2] Portzehl *et al.* (1950). [3] Mommaerts (1951a). [4] Miller *et al.* (1952). [5] Laki and Carroll (1955). [6] Holtzer and Lowey (1956). [7] Hasselbach and Schneider (1951). [8] Hanson and Huxley (1957). [9] H. H. Weber (1950). [10] Mommaerts (1951a). [11] Tsao (1953a). [12] Mommaerts (1952a). [13] Tsao *et al.* (1951). [14] A. G. Szent-Gyorgyi (1953).

to 8.2×10^{-13}, suggesting that aggregation has taken place, and with longer incubations at this temperature the change becomes irreversible. Holtzer and Lowey (1956) have also recently found a value of 530,000 for the molecular weight of myosin, the values of S_{20}^{W} that they obtained being 6.38 to 6.43×10^{-13}. Parrish and Mommaerts (1954) reported a value of $S_{20}^{W} = 6.66 \times 10^{-13}$ with $D_{20} = 1.05 \times 10^{7}$, leading to a molecular weight of 650,000. Miller *et al.* (1952) had previously reported $S_{20}^{W} = 6.2 \times 10^{-13}$ for preparations of myosin from rabbit, rat, and mouse. Higher values of sedimentation constant were found by Portzehl (1950) and taking $S_{20}^{W} = 7.7 \times 10^{-13}$, $D_{20} = 0.9 \times 10^{-7}$, a value of 821,000 was obtained for the molecular weight. This was in good agreement with the value of 840,000 obtained from osmotic pressure measurements (Portzehl *et al.*, 1950).

An estimate of the molecular weight of myosin can be made in a rather different way. Myosin can be fragmented by trypsin (Gergeley, 1950, 1951, 1953; Perry, 1951; Mihalyi, 1953; Mihalyi and Szent-Györgyi, 1953a, b) into two different components having molecular weights of 236,000 and 96,000 (A. G. Szent-Györgyi, 1953), there being two of the 96,000 molecular weight fragments (light meromyosins, LMM) liberated for each of the 236,000 molecular weight fragments (heavy meromyosin).[1] The heavy meromyosin fragments (HMM) contains all the ATPase activity of the original myosin and also the ability to combine with actin. No appreciable quantity of any other component is released at the same time. The molecular weight of myosin must therefore be 428,000, or some multiple thereof, if all the original molecules of myosin are identical. The experimental values, in which the multiple is "more than one but a little less than two," may reflect a tendency of the "monomeric" myosin molecules to form dimers, as indeed several authors have suggested, and the latest values for the molecular weight suggest that under suitable conditions, this tendency can be reduced but not completely eliminated.

The dimensions of the myosin molecule are rather uncertain. The axial ratio is high, probably too high for reliable estimates of length and breadth to be made from it. Portzehl (1950) and Portzehl *et al.* (1950) have reported an axial ratio of 94 and a molecular weight of 821,000 from sedimentation-diffusion studies, and a molecular weight of 840,000 and an axial ratio of 128 from osmotic pressure measurements. Taking a mean axial ratio of 100 and a mean molecular weight of 830,000, dimensions of 2300 A. × 23 A. were calculated. Mommaerts (1951a) has deduced a value of 1500±100 A. as the molecular length from light-scattering experiments. Holtzer and Lowey (1956) have calculated that their light-scattering observations predict dimensions of 1650 × 27.9 A. for molecules in the form of rigid rods. As these measurements may refer to myosin which had associated to some extent, some uncertainty remains as to the length of an individual molecule. Indeed, it is highly improbable that such a molecule would have the same shape as a simple long, narrow rod or ellipsoid, and such measurements as these can in any case give only a general indication of its dimensions.

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[1] See also Lowey, S., and Holtzer, A. (1959). *Biochimica et Biophysica Acta* 34, 470.

b. Structure of myosin. Some information about the internal structure of myosin may be found from the dimensions of the myosin fragments reported by A. G. Szent-Györgyi (1953). The LMM fraction can be crystallized in the form of long needles which show, in the electron microscope, a periodicity of 424 A. (Philpott and Szent-Györgyi, 1954). The "hydrodynamic" dimensions of these molecules are 620 × 18.8 A., but the electron microscope period is probably a better measurement of length. The hydrodynamic dimensions of the HMM molecules are 492 × 32.8 A. Lauffer and Szent-Györgyi (1955) on the basis of sedimentation data have suggested that the most probable arrangement of the subunits in the myosin molecule (assuming their shape there is the same as when they are released) is end to end, i.e., two "lights" and one "heavy" in series, with their long axes parallel to the long axis of the composite myosin molecule; this would lead to a molecular length of 1300–1600 A. for myosin "monomers."

Oriented samples of myosin give an X-ray diffraction diagram of the α-protein variety, and a similar diagram is given by whole muscle, suggesting that the elongated myosin molecules are arranged there with their long axes roughly parallel to the fiber axis (see Section II, B, 1, a for references). A similar but superior diagram is given by oriented samples of light meromyosin (Cohen and Szent-Györgyi, 1957). One is therefore led to the conclusion that the light meromyosin units in myosin are arranged with their long axes parallel to the long axes of the myosin, and hence parallel to the fiber axis of the muscle. Furthermore, it is striking that the 424-A. period seen in light meromyosin crystals should be so similar to the ~400-A. axial distance which separates similarly oriented cross bridges on the primary filaments seen in the electron microscope. These thick filaments must contain a number of myosin molecules in parallel, and the cross section of the filaments (diameter ~100–110 A.) could account for ten to twenty of them. The length of the thick filaments is about 15,000 A., so they must contain a number of myosin molecules in series as well; there is room for ten to twenty sets of myosin molecules end to end, so that the total complement would be about 200 myosin molecules per filament. One might speculate that the "backbone" of the thick filaments was made up of the LMM units whose length controlled the axial period in these filaments, while the HMM units, attached to the "lights," projected out sideways and formed the bridges seen in the electron microscope. Certainly one would expect the bridges to contain HMM, for it is that fragment which can combine with actin.

c. Myosin content of muscle. This will be an appropriate point at which to consider the actual quantity of myosin present in muscle. Taking a myosin content of 36% of the total protein (Hanson and Huxley, 1957), a protein content of 20% by weight of the whole muscle, and a value of 424,000 for the molecular weight of myosin, one may calculate that there should be approximately 10.8×10^{16} molecules of myosin in 1 cc. of muscle. If we assume that the filaments occupy the whole cross section of the muscle (which will lead to an overestimate of their number), that the thick filaments are in a hexagonal array 455 A. apart at rest length, and that the sarcomere length is then 2.3 μ, then the number of thick filaments in 1 cc. of muscle may be shown to be approximately 2.5×10^{14}. Hence the number of myosin molecules in each filament should be approximately 432.

The number of cross bridges which appear on each thick filament in the electron microscope is approximately 216 (about eighteen sets of six bridges in each half of the A-band). Now in the first place, it is of great interest that these two numbers, arrived at by such different routes, and involving such astronomical intermediaries, should be so similar (the exact factor of 2 between them is of course accidental). It indicates that the identification of the cross bridges with linkages between actin and myosin molecules is in at least approximate agreement with the known myosin content of muscle. However, we may enquire why the agreement is not more accurate than is actually found. There seem to be two possibilities. One is simply that the observed bridges in fact each correspond to two myosin-actin linkages. The other possibility is that the estimates of the myosin content of muscle are inaccurate by a factor of 2. That is, that the "myosin" precipitated from the muscle extract contains only 50% of the component which after further purification is found to behave as "pure" myosin.

The thick filaments seem to have six bridges spaced out fairly evenly for each 400 A. of their length. That is, the bridges which project out in the different directions in any 400-A. interval do not all lie in register. This would suggest that the myosin molecules are not arranged in register in the thick filaments either, an idea that is further supported by the tapered appearance of those filaments at their ends (H. E. Huxley, 1957). Such an appearance would arise automatically at the ends of a rod built out of long molecules arranged in a staggered fashion as shown in Fig. 23. If the molecular length was 1600 A., and if each molecule contained one heavy meromyosin bridge, then the main part of the filaments would contain 24 molecules in parallel. If the molecular length were 800 A., then the filaments would contain 12 molecules in parallel.

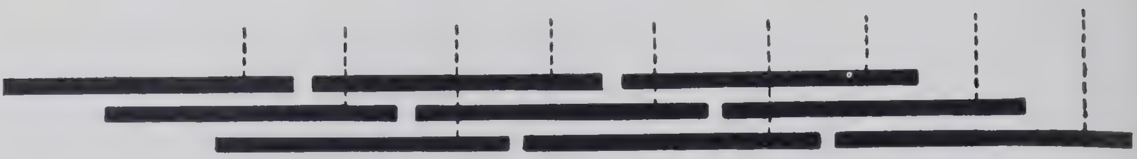

FIG. 23. Diagrammatic representation of "staggered" array of long molecules, showing how a filament built up in this way will have tapered ends.

2. *Actin*

a. Size and shape. Actin seems to be able to exist in a number of different forms—as a monomer, as a dimer, or as a structure which displays many of the properties of a high polymer, but which has other properties which are very unexpected and which we will consider presently. The molecular weight of the monomeric unit has been estimated by Tsao (1953a), using the fluorescence depolarization technique (G. Weber, 1952), as 70,000. Mommaerts (1952a) gives a value of 57,000 derived from light-scattering measurements. Information on dimensions are scanty, as the monomers are stable only in salt-free solution where local charge effects will complicate the hydrodynamic behavior. H. H. Weber (1950) suggests a very approximate axial ratio of about 5 from sedimentation data; this would correspond to a rod with dimensions of 28 × 140 A. Viscosimetric measurements (Tsao, 1953a) indicate an axial ratio of ~ 12, corresponding to dimensions of 290 A. × 24 A. for a prolate ellipsoid of revolution; when actin is in the dimeric form, the viscosity data indicate an approximate axial ratio of 22, indicating an end-to-end association. The dimers show the expected molecular weight of 140,000 by the fluorescence method (Tsao, 1953a).

b. Structure of F-Actin. At higher salt concentrations (say 0.1 *M* KCl), an interaction between the small actin units occurs; the viscosity of the solution becomes very high (Straub, 1942, 1943a), and it exhibits both flow and spontaneous birefringence, and thixotropy. The sedimentation constant becomes very high ($S_{20} = 50$ or more) (Portzehl *et al.*, 1950; Johnson and Landolt, 1950), and if oriented specimens of dried actin are examined in the electron microscope (Rosza *et al.*, 1949) or by X-ray diffraction techniques (Astbury *et al.*, 1947; Cohen and Hanson, 1956), they show clear evidence of a regular axial periodicity repeating over distances much longer than the individual monomers or dimers. These observations obviously suggest that long polymers of actin are present in the solutions. However, the work of Tsao (1953a, b) shows that we need to consider all this evidence rather circumspectly.

By coupling a fluorescent dye onto a protein molecule and measuring the degree of depolarization of the fluorescent radiation excited by polarized light, it is possible to compute the mean rotational relaxation time of the system. For protein molecules with an axial ratio greater than 10 and a molecular weight of the order of 100,000 or more, the relaxation time about the short axis is too long to be measured and only the relaxation time about the long axis of the molecule is obtained. From this and the coefficient of viscosity, the molecular weight can be computed.

Using this technique, Tsao (1953a) found that F-actin behaved as though its molecular weight were 140,000, i.e., the same as that of the dimers. This surprising result might be explained in two ways. It might be supposed, as Tsao suggests, that the dimers interact with each other to form "structures in solution" which give rise to the polymerlike behavior, but in which the kinetic units are still dimers. A slightly different hypothesis would be to suppose that the dimers are linked together by permanent bonds about which there is sufficient freedom of rotation for the dimer to turn about its long axis and produce the observed fluorescence behavior. So far, no X-ray diffraction results have been reported on wet gels of actin, so that we do not have any direct structural information about F-actin in solution.

X-Ray studies on dry gels of actin and on dried muscle have, however, been very illuminating. Using dry specimens of the tinted portion of *Venus* adductor muscle, Selby and Bear (1956) have obtained excellent diffraction patterns which they have interpreted in considerable detail. These patterns have strong general similarities to the much less detailed patterns given by dry oriented actin and by various dried striated muscles from vertebrates, and it was considered probable that they arose mainly from an actin component in the *Venus* adductor muscle. These patterns could be interpreted in terms of either a net, i.e., a flat array of nodes (regions of higher or lower electron density), or of a helical structure obtained by rolling the net up into a cylinder. A diagram of the proposed net structure is shown in Fig. 24. The net or helix contains fifteen nodes in the unit cell which 406 A. long. The width of the unit cell in the net is 82 A.; on the helical interpretation, the diameter of the cylindrical shell through the nodal centers would be about 50 A. This value is very similar to the diameter of the secondary filaments observed in rabbit psoas muscle in the electron microscope. The proposed actin structure shows a very pronounced pair of nodal rows (shown by the dotted lines in the diagram) which form a double helix in the helical interpretation. This helix with fifteen nodes in 406 A., would have a threefold axis of symmetry when viewed end on (see Fig. 25). It would therefore be a very suitable structure for forming cross links to three myosin filaments placed symmetrically about it. Only one-fifth of the nodes (if we were to identify them as actin molecules) would be linked at any one time, if the bridges seen in the electron microscope represent single actin-myosin

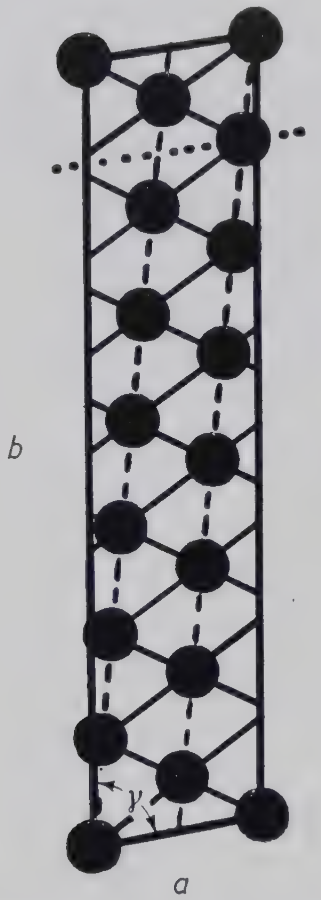

FIG. 24. The actin two-dimensional net cell. Cell elements a, b and γ are 82 A., 406 A. and 82 A., respectively, for dry samples. From Selby and Bear (1956).

linkages. The actin filaments would need to rotate about their long axes by 180° each time they moved along a distance of 406 A.

In the electron microscope, an approximately 400 A. period is observed both in the A-bands, and also in the I-bands, where actin alone (or at least actin without myosin) is believed to be present. This suggests, in combination with the 400-A. X-ray period observed on living material, that in muscle actin can exist in the form of stable high polymers, perhaps in combination with another protein. Indeed, actin is so tightly bound to the structure of the muscle that quite extreme methods are needed to extract it—acetone drying (Straub, 1942), or 0.6 *M* KI treatment (A. G. Szent-Györgyi, 1951b), or butanol treatment followed by acetone drying (Tsao and Bailey, 1953). A pattern corresponding to the arrangement of nodes described above has not so far been seen in the electron microscope, but this may merely be because the nodal structure does not stain in a specific way. The 400 A. period could arise if we were simply to see the "crossover points" in the two-chain structure.

c. Actin content of muscle. The quantity of actin which can be extracted from whole muscle amounts to about 14% of the total protein

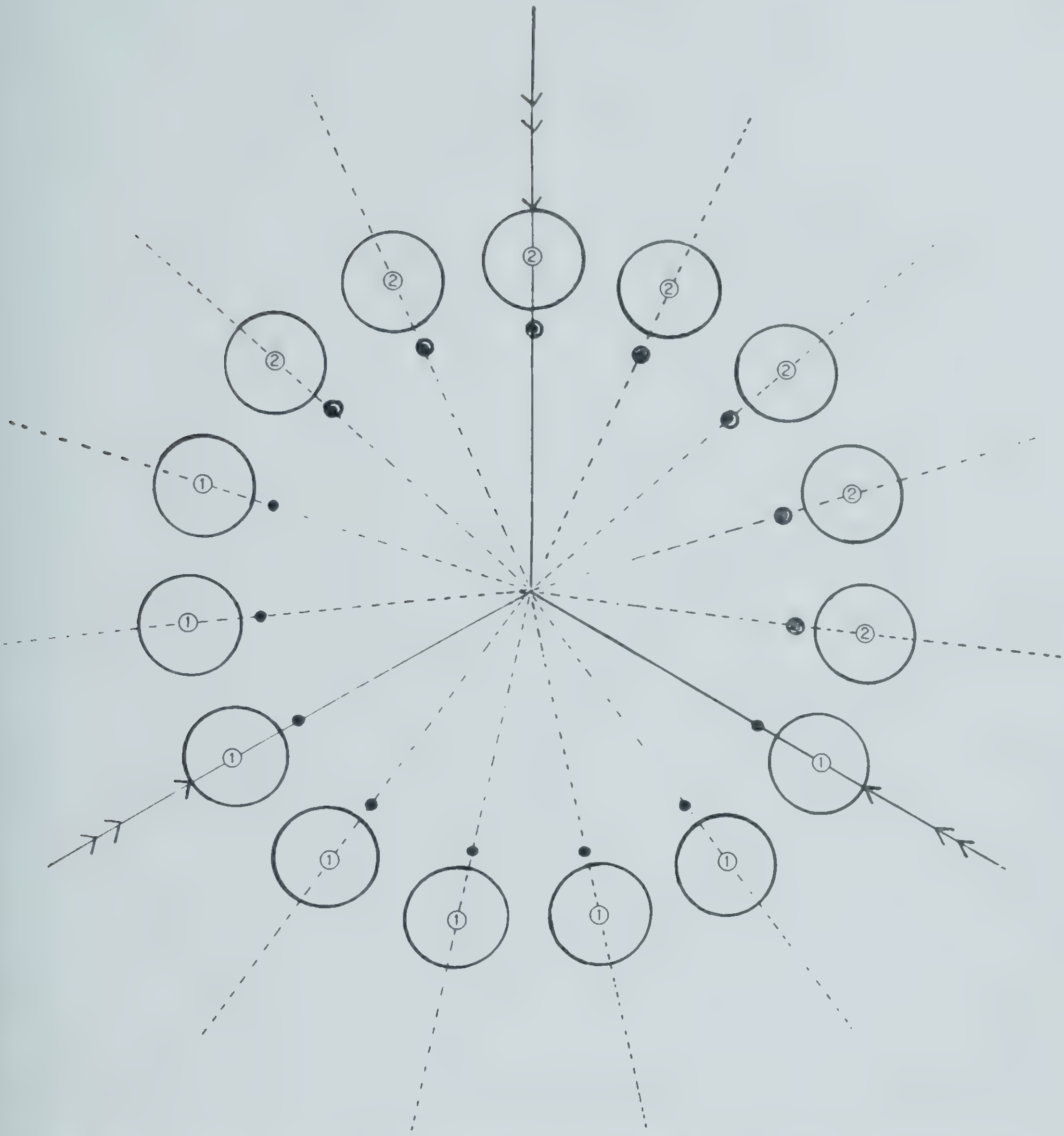

FIG. 25. End-on view of actin filament according to helical interpretation of X-ray data. Note threefold axis of symmetry. The nodes numbered *1* and *2* correspond to the projections of the nodes lying on the two dotted lines in Fig. 24.

content (Hasselbach and Schneider, 1951); the quantity of protein in the secondary filaments is about 24% of the total muscle protein (H. E. Huxley and Hanson, 1957; Hanson and Huxley, 1957). It seems that either the actin extraction is incomplete, or that a second protein is present there, or both. If the secondary filaments were built out of the helical arrangement of nodes described by Selby and Bear, and if each node represented an actin monomer, then the quantity of actin present in muscle should be 16.5% or 20.25% of the total muscle protein, taking a molecular weight of 57,000 and 70,000, respectively. There are not unreasonable values, and in each case, a little extra protein besides actin has to be present to make up the 24%. If the secondary filaments contain 24% of the total muscle protein, then their diameter must be about 70 A. This is a little greater than the value of 50–60 A. observed in the electron microscope on fixed and stained material.

The little information available on the shape of the actin monomers does not offer any specific clue as to their relation to the nodes in the X-ray structure. But it is interesting to note that in a two-chain structure to which three cross links are attached every 400 A. in the way we have described, one chain must have two links to it and the other only one.

3. *Tropomyosin*

a. Size and shape. This third structural protein of muscle, characterized by Bailey (1946, 1948) again has properties which would lead it to be classified as a fibrous protein. It forms viscous, nonthixotropic solutions which show a large and characteristic drop in viscosity when the ionic strength is increased (to 0.05–0.1 μ). The best values for the molecular weight (osmotic pressure measurements in 6.67 M urea or in 0.01 N HCl or NaOH) lie around 53,000 (Tsao *et al.*, 1951); the higher values reported earlier have been ascribed to spontaneous aggregation which still occurs even in strong salt. From the histidine content, the minimum molecular weight should be 54,600. Viscosity measurements indicate a very high axial ratio, given as 26–31 in 1.1 μ salt, and 45–53 at $\mu=0.1$. If the polypeptide chain in this molecule is all in the α-configuration, with a residue spacing of 1.5 A., then the total chain length would be 683 A. (455 residues in the monomer). If the chain is looped around on itself so as to give a molecular length of 340 A., then the axial ratio given above, would correspond to a diameter of 11–13 A., which the authors point out is in fair agreement with the average diameter of two α-helixes placed side by side (about 15 A.). Of course, the real structure may be a little more complicated than this.

b. Tropomyosin content of muscle. The amount of tropomyosin extracted from muscle by the original techniques has been estimated as about 2.5% of the total dry weight, but recent results by Perry and Corsi (1958) suggest that the total amount present may be substantially higher, perhaps as high as 10–12%. The location of tropomyosin is not known. However, Perry and Corsi (1958) have shown that tropomyosin and actin tend to be leached out in constant proportions from isolated fibrils stored in buffer, although the absolute amounts may vary considerably; they suggest that actin and tropomyosin may hold each other in place in some way inside the muscle. Perhaps tropomyosin is the extra material which, as we have seen, may be present in the secondary filaments, and to which actin may be attached.

B. *Biochemical Properties of the Muscle Proteins*

1. *Physicochemical Behavior*

a. Extraction of myosin. Myosin can be extracted from minced fresh muscle by strong salt solutions ($\mu > 0.4$–0.5). The solutions most commonly employed are known, after their originators, as Guba-Straub (1943) solution (0.3 M KCl, 0.15 M phosphate buffer, pH 6.5) and Hasselbach-Schneider (1951) solution (0.47 M KCl, 0.1 M phosphate buffer, 0.01 M sodium pyrophosphate, pH 6.5). The latter solution, it should be noted, contains pyrophosphate, and with it, it is possible to extract myosin virtually uncontaminated by actin. The other solution always extracts a certain amount of actin, the amount becoming larger with longer periods of extraction. If the muscle is allowed to go into rigor before extraction with Guba-Straub solution, then very little myosin is extracted from it (Banga and Szent-Györgyi, 1942; Erdos, 1943). Glycerinated muscle, too, resists extraction by either of the solutions described above, but myosin can be extracted very satisfactorily by Hasselbach-Schneider solution fortified by the addition of 10^{-3} M $MgCl_2$, or by Guba-Straub solution to which 5×10^{-4} M ATP has been added (Hanson and Huxley, 1953). Another very satisfactory extracting medium has been found to be 0.6 M KCl, 0.1 M phosphate buffer, 0.01 M sodium pyrophosphate, 10^{-3} M $MgCl_2$, pH 6.5 (H. E. Huxley and Hanson, 1957).

Thus it seems that an ionic strength $> 0.5\,\mu$ and the presence of either ATP (as in fresh muscle) or pyrophosphate and magnesium is required to extract the myosin, although myosin itself once having been extracted will go into solution at $\mu \sim 0.2$–0.3. Myosin in muscle appears to be bound into the structure by links which require ATP or pyrophosphate, as well as high ionic strength, to break them. Links between actin and myosin would behave in this way. It is therefore natural to suggest that the extractability properties of myosin are controlled by the tendency of the myosin in the thick filaments to form cross links to the actin in the secondary filaments, which themselves are strongly resistant to extraction. The extraction of some actin under conditions where one would expect some of these links to have formed could be explained if myosin molecules, passing into solution, could break away part of a secondary filament to which they had become attached. It is also possible, of course, that the myosin molecules in the thick filaments are held in place by links to some other protein in these filaments.

Myosin can be almost completely precipitated if the ionic strength of the solution in which it is dissolved is reduced to $0.05\,\mu$ or lower, and this method is used during the purification of myosin to separate it from other muscle proteins extracted simultaneously. If actomyosin is also present, this can be precipitated separately at $\mu = 0.15$ by a suitable amount of ATP (A. G. Szent-Györgyi, 1951b).

b. The "polymerization" of actin. We have already referred to the fact that actin can exist in two forms which are known as G-actin and F-actin; and we have seen that the F-actin structure might consist of G-actin units (which actually seem here to be dimers of the individual actin molecules of ~ 70,000 mol. wt.) joined together lengthwise by bonds about which there is some freedom of rotation. The G→F transformation assumed great importance and interest when it was shown by Straub and Feuer (1950) that ATP was involved in it. Preparations of G-actin were found to contain bound ATP in amounts corresponding to two ATP molecules per 70,000 mol. wt. unit of actin; this was transformed into bound ADP when polymerization took place, inorganic phosphate being liberated. The depolymerization of F-actin (by dialysis against very low concentrations of salt) was found to be reversible only in the presence of ATP. In its absence G-actin-ADP was produced instead of G-actin-ATP; and the former would not polymerize. The presence of magnesium appears to be essential for polymerization, and it was suggested that the nucleotide acts as the prosthetic group of actin forming the linkages responsible for polymerization through the mediation of divalent cations. These observations were, in general, confirmed by a number of other workers (Laki and Clark, 1951; Mommaerts, 1951b, 1952b; A. G. Szent-Györgyi, 1951a).

Pettko and Straub (1949) found 0.3 mg. of ATP or ADP per gram present in fresh muscle in a form inaccessible to enzymes. This corresponds approximately to 2 moles of nucleotide for every 70,000 gm. of actin, assuming an actin content of 2.8% of the total wet weight. Perry (1952b) found that isolated myofibrils contained bound ADP (but not much ATP) in amounts corresponding to there being 1 mole of ADP per 74,000 gm. of actin. These results suggest that the actin in muscle contains the nucleotide in a bound state, and that the actin is present in the F-form.

Tsao's observations on the behavior of actin have already been mentioned briefly, and we must now return to them in greater detail. He found that G-actin in solution in water was normally present in the form of dimers. Under some conditions (e.g., presence of ADP or of Versene with temperature >21° C.), the monomeric form seemed to be favored. However, it is clear that the G-actin solutions which have been shown to contain bound ATP and which polymerize with the liberation of inorganic phosphate correspond to solutions shown by Tsao to contain actin which is already in the dimeric form. Thus the monomer-dimer transformation in actin is distinct from the G→F transformation in which Straub and Feuer showed nucleotide to be involved. It seems that it is

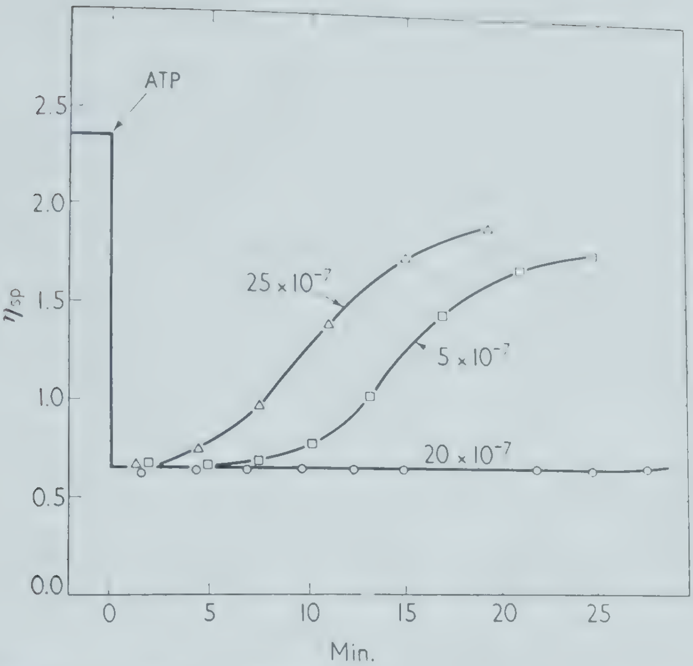

FIG. 26. Effect of addition of ATP on the viscosity of actomyosin solutions containing 12.5 mg. protein in 5 ml.; temperature 0° C. From Mommaerts (1948).

in the coupling together of the dimers that bound ATP is dephosphorylated.

c. Interactions between actin and myosin. When solutions of myosin and actin in 0.5 *M* KCl are mixed together, a large rise in viscosity takes place (Straub, 1942, 1943b). This rise can be reversed by the addition of ATP; the viscosity falls to a value corresponding to that of the two components acting independently and then slowly recovers as the ATP is dephosphorylated by the myosin (Mommaerts, 1948) (Fig. 26). The usual interpretation of these phenomena is that a combination takes place between the myosin molecules and the F-actin polymers, causing the latter to become heavier, more rigid, and perhaps also more cross-linked with one another; and that this combination can be broken by ATP.

Bailey and Perry (1947) showed that the presence of intact SH groups on the myosin is necessary both for actomyosin formation and for ATPase activity, and they suggested that ATP might compete for the site at which actin is bound.

Snellman and Erdos (1949), Portzehl *et al.* (1950), and Mommaerts (1951c) found that the myosin peak could be seen in the sedimentation diagram of actomyosin solutions to which ATP had been added; otherwise it was absent. More recently Annemarie Weber (1956) investigated the sedimentation behavior of solutions of "natural" actomyosin (24-hour muscle extracts) spun at 100,000 *g* for about 3 hours, in the presence and in the absence of ATP. In the presence of ATP, centrifugation produced a small dense pellet and a water-clear solution; the protein in the supernatant was on the average about 56% of the total protein originally present and displayed all the properties of pure myosin. Thus the actomyosin had clearly dissociated under these conditions. In the absence of ATP, the water-clear solution found on centrifugation contained only 2–3% of the total protein, the rest of it being present in a lower layer of turbid solution and a large opaque precipitate, both of which behaved as actomyosin. Thus, without ATP, practically no dissociation took place.

A different view of the changes produced by the interaction of ATP with actomyosin or myosin has been put forward by Blum and Morales (1953). Using the technique of light scattering, they investigated the behavior of extracts of rabbit muscle, made with Weber-Edsall solution (the extraction times being either 5 or 24 hours), when treated with ATP. The results were analyzed by the Zimm plot method (1948). The particle weights varied from sample to sample, in the range 4.3 to 31.3×10^6, but no change in particle weight in a given sample was detected on the addition of ATP. The results indicated instead an increase in the radius of gyration of the particles when ATP was present.

Gergeley (1956), also using the light-scattering technique, has carried out the experiment in a somewhat different way and has obtained results more in line with the "dissociation" hypothesis. Larger particles were removed from the solution immediately before the measurements were taken. The particle weights before ATP was added lay in the range 10 to 20×10^6 and they were reduced to 2 to 6×10^6 by the addition of ATP. Gergeley suggests that a small number of very large particles in a preparation would dominate the light-scattering pattern at small angles, and that if *these* particles did not dissociate, a Zimm plot extrapolation to zero angle and zero concentration would show an unchanged molecular weight irrespective of what had happened to the smaller particles.

Both A. Weber's and Gergeley's experiments prove quite clearly that ATP can cause dissociation of *some* actomyosin complexes. That a genuine change in shape may occur in the large complexes which are not dissociated by ATP has not been disproved, however, and Morales (personal communication) considers that this is the case.

At lower salt concentrations ($\sim 0.3\,\mu$), the addition of ATP to an actomyosin solution causes the protein to form a precipitate which shrinks down to a dense plug (A. Szent-Györgyi, 1943). It has always been argued by Szent-Györgyi that this phenomenon was very closely related to contraction, and that the contraction was isodimensional simply because the plug was not oriented. When the actomyosin was oriented in the form of threads, ATP produced an isodimensional shortening (see Sections I, E and II, C, 1, b).

It seems then that the action of ATP at high salt concentration can cause actomyosin to dissociate, while at a lower salt concentration it causes a suitably oriented complex of actomyosin to shorten. It is possible to argue that the first action of ATP, in both cases, is to break the link between actin and myosin; and that in high salt concentrations, the actin and myosin molecules then move apart and interaction between them ceases, while in the gel form, the actin and myosin molecules, though disconnected, do not separate, that other changes are sterically possible, and that these lead to contraction when the ATP is split. This view is a plausible one, and we have seen that it enables us to give a reasonable account of some of the properties of muscles and of fiber models in terms of our structural model. But the exact changes which occur in the actomyosin complex in the presence of ATP are largely unknown, and we should guard against thinking about them in terms which imply more than we actually know.

Tsao (1953b) has used the fluorescence depolarization method to study the interaction between actin, myosin and ATP. The kinetic units in free F-actin are dimers, and we have already noted that this result might perhaps be reconciled with the high-polymer-like behavior of F-actin if the dimeric units had freedom of rotation about their long axes, like a string of oblong beads. Now the weight of myosin molecule is so large that if fluorescently labeled actin dimer became rigidly fixed to it, a very large increase in the apparent molecular weight of the latter should be measurable by the fluorescence method. But again, the experimental result was completely bizarre and unexpected; it was found that in the presence of myosin, labeled actin behaves as *monomers* with a molecular weight of about 70,000, despite the high viscosity and flow birefringence of the system. When ATP is added, so as to "dissociate" actin and myosin, the fluorescence depolarization behavior changes instantaneously to that appropriate to dimers, and then recovers as the ATP is split (Fig. 27).

This is a very remarkable result indeed, and in this author's opinion it is the most important uninterpreted clue that we have to the mechanism of muscular contraction. The high viscosity, flow birefringence, and sedimentation behavior of the actomyosin solutions indicates that they contain long polymers. If the dimers of actin have been split into monomers, then the integrity of the polymers would have to be maintained by the myosin, perhaps by each myosin molecule combining with two molecules of actin; the actin monomers would have to have freedom of rotation about their bonds to the myosin, which would have to be colinear with the actin-actin bands. It would be interesting to know whether the dimer-monomer transformation is produced by myosin preparations which show the lower value of molecular weight (Section III, A, 1, a), or by heavy meromyosin.

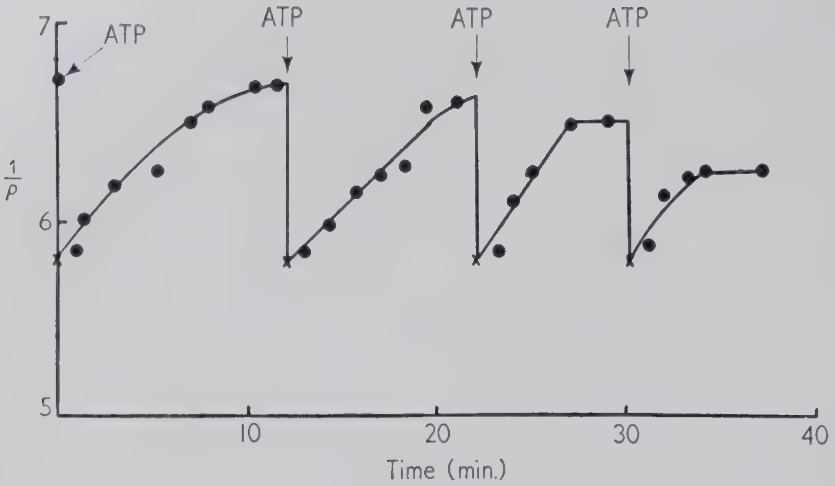

FIG. 27. Effect of ATP on the fluorescence-depolarization of F-actomyosin, in 0.5 *M* KCl + 0.006 *M* phosphate, pH 7.0, 26.0° C. From Tsao (1953b). (By courtesy of *Biochimica et Biophysica Acta.*)

This particular technique has so far not been applied very widely, and one really needs more experience with it before one can interpret the results with certainty. But they do seem to suggest, as a very minimum, that a very dramatic change might take place in the internal structure of an actin filament when it became cross-linked with myosin, and this possibility should be constantly kept in mind when considering the detailed mechanism of contraction.

2. *Enzymatic Behavior*

a. Enzymatic properties of myosin and actomyosin. Myosin and actomyosin have the ability to catalyze the hydrolytic cleavage of the terminal phosphate group of ATP (Engelhardt and Ljubimova, 1939). The rate at which this reaction takes place is profoundly influenced by the presence or absence of divalent cations, like Ca^{++} and Mg^{++}, by salt concentration, and by pH, and great differences exist in the effect these various factors produce on purified myosin, and on myosin in the presence of actin.

The ATPase activity of pure myosin is activated by calcium (Bailey, 1942) and has pH optima in the alkaline region and around pH 6.4 (Mommaerts and Green, 1954). The variation in activity with pH under various ionic conditions is shown in Fig. 28, taken from the last-mentioned paper. It has been suggested that the increase in activity at high pH is due to the increase in concentration of (OH) ions (Ouellet *et al.*, 1952). The presence of Mg^{++} has a strong inhibitory effect; with a $Mg^{++}:Ca^{++}$ ratio of 0.04 : 0.05, activity is reduced to one-half, and with a $Mg^{++}:Ca^{++}$ ratio of 0.5 or more, activity is reduced to 5% at pH 6.4 and to 2% at pH 9.2.

Mommaerts and Green obtained maximal activity at pH 6.4 with

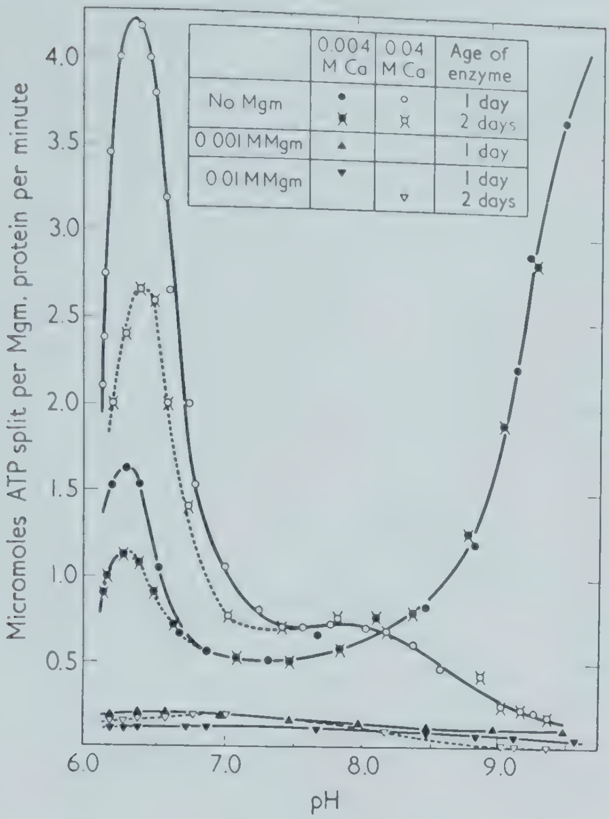

FIG. 28. Effect of pH on ATPase activity of myosin in 0.15 *M* KCl in the presence of divalent ions. Myosin 1 day after crystallization, unless otherwise specified. From Mommaerts and Green (1954). (By kind permission of the *Journal of Biological Chemistry*.)

0.04 *M* Ca^{++} in 0.15 *M* KCl, the rate of breakdown of ATP then being 5 μ moles per minute per milligram enzyme at 27° C. It is interesting to express this rate in another way: we will take a molecular weight unit of 424,000 for myosin (two light meromyosins, plus one heavy meromyosin, which is the enzymatically active fragment) and calculate how many molecules of ATP are split per second by such a unit. Mommaerts and Green's values lead to a figure of 35. This may be compared with the figure of 100 molecules of ATP which we calculated (Section II, C, 5), from the mechanical and thermal data, to be split at each of the cross bridges per second when a muscle was shortening at a maximum velocity of ten muscle lengths per second. The two figures are clearly of very similar magnitudes.

In a muscle, however, as Mommaerts (1947) has pointed out, the magnesium: calcium ratio (0.012 *M* : 0.006 *M*) (Perry, 1956) is such that one would expect the activity of myosin ATPase to be very largely inhibited; but it would be strange to have a built-in enzyme which could not function, and the contraction of glycerinated fibers is *accelerated* by Mg^{++}. The view has gradually developed now over a number of years that the solution to this paradox lies in the fact that the characteristics of the ATPase activity of myosin are greatly modified when it is present in the form of an actomyosin gel (Biro and Szent-Györgyi, 1949; Hasselbach, 1952), or in myofibrils (Sarkar *et al.*, 1950; Perry, 1951). At low ionic strength, these preparations show magnesium-activated ATPase activity with a pH optimum around 7.0. At higher ionic strengths (e.g., 0.5), where the actomyosin is present as a sol, the ATPase activity of actomyosin (Hasselbach, 1952) or of fibrils (Steinbach, 1949) once again resembles that of pure myosin, and is calcium activated.

These results indicate very strongly that it is the combination with actin which changes the influence of magnesium from an inhibitory to an activating one, and which shifts the pH optimum from 6.4 to 7.0. In high salt concentration, when, as we have seen (Section III, B, 1, c) ATP dissociates the actomyosin complex, the myosin then splits the ATP "on its own," and the characteristics of that ATPase are those of pure myosin. In low salt concentration, when ATP produces, not dissociation but contraction, the ATPase activity of the myosin is dominated by the presence of the actin.

These observations have a much deeper significance than that of merely resolving a slight complication in the nature of the divalent cation interactions. What they show is that in the presence of magnesium, the rate at which ATP is split by enzyme sites on myosin is low when actin is absent, and is high when actin is present. It is difficult to see how this effect can occur unless, at the molecular level, an actin monomer or dimer is exerting a very specific influence on the myosin ATPase; and this must involve a specific steric arrangement of the two molecules. We are therefore in possession of a very definite clue concerning one of the most basic features of muscular contraction—the fact that the energy release is to some extent controlled by the mechanical state of the system.

The detailed kinetics of the ATPase systems have been the subject of a number of investigations recently. It was shown by Ouellet *et al.* (1952) that under stationary conditions myosin ATPase obeys Michaelis-Menten kinetics according to the following scheme.

$$M + S \underset{k_1}{\overset{k_1}{\rightleftharpoons}} M\ S \overset{k_2}{\rightarrow} M + P \quad V = \frac{V_{\max}}{1 + \frac{k_m}{(S)}}$$

$$k_m = \frac{k_{-1} + k_2}{k_1}$$

$$V_{\max} = k_2\,(MS)$$

The most interesting features of the system are however, the kinetics of the mechanical effects associated with the chemical reactions. The changes in the light scattering by actomyosin solutions provide a possible way of studying these. The results to date have been very complex, and as we have already seen, some controversy exists over the interpretation of the light-scattering measurements. It would probably be premature at present to try to relate these findings to the detailed mechanics of muscle, although this approach is one of the most promising ones; reference should be made to the original papers for details (Morales *et al.*, 1955; Tonomura, 1956; Tonomura *et al.*, 1952).

b. Relaxing factors. i. The evidence. The enzymatic behavior of fibril preparations and of actomyosin is further complicated by the existence of the so-called "relaxing factors," which inhibit ATPase activity and which seem to be responsible for the ability of unstimulated living muscle to maintain its resting, relaxed state in the presence of ATP.

Marsh (1951, 1952) investigated the changes that took place in the fiber volume of a brei of broken muscle fibers when it was centrifuged immediately after preparation from fresh muscle. He found that the volume showed a sharp decrease after a length of time which varied somewhat from preparation to preparation but which was usually of the order of an hour. He found that this volume decrease could sometimes be reversed by the addition of ATP, although on other occasions a similar addition produced only a volume decrease. If the fibers were well washed, the addition of ATP always produced a volume decrease. A factor appeared to be often present in the supernatant of the original muscle brei which was responsible for the swelling and which, when added to washed fibrils, would cause them, too, to swell in the presence of ATP. Marsh showed that the ATPase activity of the preparations was decreased tenfold in the presence of this "relaxing" factor.

These experiments were confirmed and extended by Bendall (1953, 1954) and by Hasselbach and Weber (1953), who showed that this factor would reverse the contraction of glycerinated fibers in ATP. Hasselbach and Weber (1953) concluded that the factor works by greatly lowering the level at which the ATP concentration becomes "over-optimal" (i.e., so high that it causes not contraction but relaxation). Bendall (1954) concluded that the factor might be myokinase, and suggested that it might exert an inhibiting action on myosin ATPase by some reaction in which pyrophosphate (which has a remarkably similar inhibiting and relaxing effect) was produced.

In addition to myokinase (which catalyzes the reaction 2 ADP→AMP + ATP) a number of other systems which will produce ATP from ADP have been suggested as possible relaxing factors. Before considering them in detail, it will perhaps clarify the situation if we say that some divergence of opinion exists as to whether the factors described exert their effect directly by themselves altering the ATPase activity in the fibrils, or whether they act by maintaining the general or local concentration of ATP inside the fibers at a high level so that some other relaxing factor, which only operates at fairly high ATP concentrations, can exert *its* effect. [When ATP is being broken down inside intact glycerinated muscle fibers immersed in an ATP solution, the rate of diffusion of ATP inward from the surrounding medium may be insufficient to maintain the concentration of ATP inside the fiber anywhere near the outside level (Biro and Szent-Györgyi, 1949; A. Weber, 1951). Thus, although the outside level may be above that at which the relaxing factor can operate, the level inside may always lie below that level, and no relaxation will take place. This situation can be avoided if the ADP produced in the fiber is rephosphorylated to ATP while still inside.]

Goodall and Szent-Györgyi (1953) found that in the presence of 10 mM CP, 4 mM ATP, 1 mM Mg^{++}, muscle fibers which had been glycerinated for a moderate period would contract at pH 7.5 but would relax again at pH 6.2–6.6. Addition of 0.8 mol. equiv. of calcium prevented relaxation. If the fibers were washed for 5 days at –20° C. in a 0.05 M sodium bicarbonate-glycerol mixture, the relaxing ability was lost. It could be restored again by incubating the fibers for 30 minutes in a dialyzed extract of fresh muscle. This work was extended by Lorand (1953), who showed that the purified enzyme, ATP-CP-transphosphorylase, prepared according to the method of Kuby *et al.* (1954a) had high relaxing activity on glycerinated muscle fibers in the presence of creatine phosphate.

Goodall (1956) has shown that carnosine phosphate will produce relaxation under similar conditions to those required by creatine phosphate, and has suggested that this phosphate compound may be present in muscle.

More recently, Moos and Lorand (1957) have investigated the phosphoenolpyruvate (PEP) system in which the following reaction occurs:

$$\mathrm{PEP} + \mathrm{ADP} \rightarrow \mathrm{PE} + \mathrm{ATP}$$

They found that fibers washed in 20% glycerol for 1 hour (after normal glycerol extraction) gave no spontaneous relaxation with ATP. However, in the presence of >2 mM ATP and magnesium, 10 mM PEP produced a striking relaxation. At lower ATP concentrations, PEP enhanced contraction. After longer washings in glycerol, a loss of relaxing effect occurred; this did not seem to be associated with the loss of PEPase from the fibers, and it could not be restored by adding PEPase to the preparation. The authors concluded that some other factors are also very probably involved in the relaxing effect.

A similar conclusion was reached by Kumagai *et al.* (1955), who separated a muscle-relaxing factor extract into two fractions, A and B, by ammonium sulfate fractionation. Fraction B contained myokinase and creatine phosphokinase. Fraction A was highly opalescent and had strong ATPase activity, suggesting it contained some of the soluble ATPase systems of the muscle. Separately, the two fractions produced no relaxation at all on fibers which had been glycerinated for longer periods than one month and then washed for several hours in 0.1 M KCl, before being treated with 5 mM ATP and 0.01 M $MgCl_2$. When applied together, however, fractions A and B produced relaxation.

The nature of relaxation has also been investigated by Bozler (1953, 1954a, b, 1955, 1956) and Bozler and Prince (1953) in an extensive series of experiments. Using fiber bundles which had been extracted in glycerol for only 2–4 days, they found that concentration $>5\,M$ of ATP would cause spontaneous relaxation, presumably because sufficient relaxing factors were still present in the fibers. The addition of 1.5 mM Ca^{++} to the medium produced contraction and at the same time a large rise in the rate of ATP splitting by the preparation took place, from 1.8×10^{-6} mole per minute per gram wet weight to 8×10^{-6} mole per minute. It is interesting to note at this point the observation by Wiercinski (1952) and by Niedergerke (1955) that the microinjection of calcium into a living, relaxed muscle fiber will produce a local contraction. Contraction also occurred if the ATP concentration was less than 5 mM. In the absence of magnesium, contraction was produced at all ATP concentrations. If fibers which had been stored in glycerol for a longer time were used, ATP was found to produce only contraction. Presumably the relaxing factors had been washed out of the fibers or inactivated in some way.

The effects of the diffusion rate of ATP on the interpretation of results obtained with different types of phosphorylating system has recently been discussed at length by Portzehl (1957) and by Briggs and Portzehl (1957). Portzehl found that the presence of the CP-CPase system alone had no effect on either the ATPase activity or the ATP-induced contraction of isolated *myofibrils* prepared from muscle which had been glycerinated for 2 weeks or more. On the other hand, both activity and contraction were inhibited by preparations of Marsh factor alone. The use of isolated myofibrils eliminated effects due to diffusion, at the ATP concentrations involved. Portzehl and Briggs argue that the real relaxing factor is not the creatine phosphate system or some other phosphorylating system, but that it is another agent altogether, which does not require the simultaneous presence of a phosphorylating system, and which extends the overoptimal ATP concentration (i.e., the range in which substrate inhibition and tension decrease occurs) to the range below $10^{-2} M$, the effect beginning at $\sim 5 \times 10^{-5} M$ at pH 6.2 and $\sim 4 \times 10^{-4} M$ at pH 7.2; in thick fibers or in fiber bundles these concentrations can only be produced inside the fibers (in the absence of a phosphorylating system) by quite high concentrations of ATP outside ($\sim 6 \times 10^{-3} M$). When a phosphorylating system is present (for instance, the CP-CPase system), overoptimal concentrations of ATP can be produced inside the fiber with lower outside ATP concentrations, and at certain ATP concentrations, the relaxation effect can become pH dependent, as found by Goodall and Szent-Györgyi (1953).

Portzehl (1957) has shown recently that the active fraction of Marsh factor preparations can be sedimented at 20,000 *g*, and must therefore be associated with quite large structures. Huxley, Hasselbach, Hanson, and Lowy (unpublished observations) have examined this sedimentable fraction in the electron microscope and have found that it consists of vesicles (1000–2000 A. in diameter) and membranous or tubular structures similar to those found (Bennett and Porter, 1953; Porter and Palade, 1957) lying between the myofibrils of whole muscle (Fig. 29).

ii. Conclusions. The general picture that emerges from these results may be summarized as follows: Fresh muscle contains some system which keeps the muscle in the relaxed state until it is stimulated; ATP is present, but the ATPase activity is low and the muscle can be extended plastically by a small force. This relaxing system probably consists of a "relaxing factor" which can prevent an unstimulated muscle from contracting (providing the ATP level is maintained), and an enzyme system which can rephosphorylate ADP to ATP. This relaxing system can be inactivated, and contraction produced, by injected calcium, but it is not known by what process physiological stimulation occurs. When the muscle is glycerinated it loses the ATP (and CP) which it contained and passes into a rigorlike inextensible condition; the relaxing factor is also leached out or inactivated, but at a much slower rate than the ATP, so that for the first day or two, the fibers will still go into the relaxed state if sufficient ATP is supplied to them. Presumably the concentration of relaxing factor is still sufficiently high to produce relaxation with the concentrations of ATP which can diffuse into the fiber. This relaxation is transformed into contraction if calcium is added. After longer periods of glycerination, relaxation can be brought about only if a phosphorylating system such as CP-Cpase is provided as well as ATP, so as to produce a high ATP concentration inside the fiber, presumably required with the now depleted amount of relaxing factor; and after even longer extraction of the fibers, a factor, present in fresh muscle extract, has to be added to the system in addition to CP and CPase, if ATP is to produce relaxation. This factor

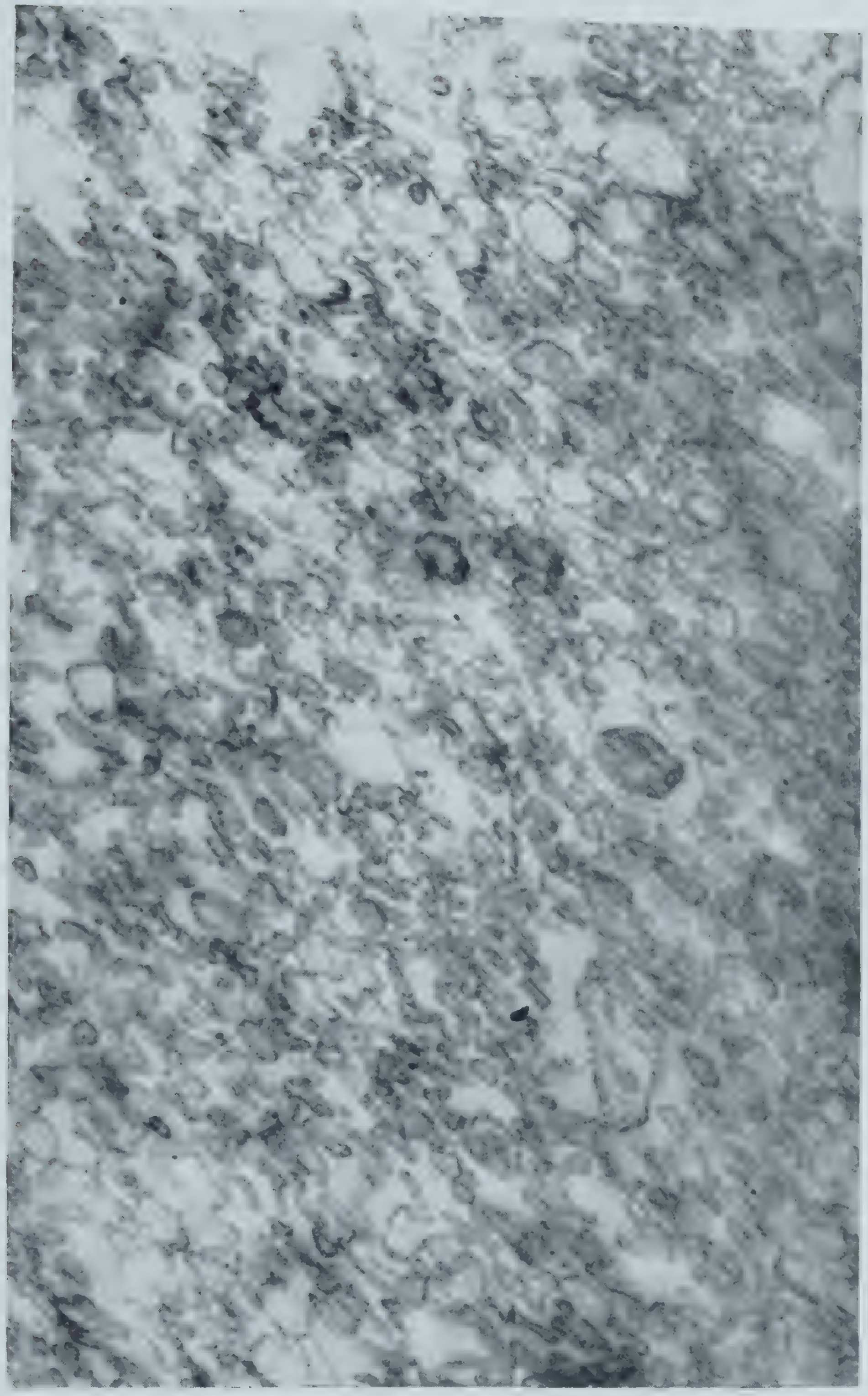

FIG. 29. Electron micrograph of section of pellet of sedimented relaxing factor. Note similarity of the particles and vesicles to the structures lying between the fibrils in intact muscle (see Fig. 3). Magnification: ×37,000.

seems to be bound to the particles and membranes which lie between the myofibrils in whole muscle.

c. ADP and contraction. Some other very interesting observations on the effect of the creatine phosphate system have been made by Perry (1954) and by Bozler (1953). Perry found that isolated myofibrils (which had been extensively washed) would contract in extremely low concentrations of ADP (2.8 to 5.6×10^{-6} *M*) if creatine phospate (5×10^{-3} *M*) and CPase were also present. In their absence, concentrations of ATP ten to a hundred times as great as the ADP concentrations referred to were required to produce contraction. Portzehl's calculations (1957) (Table I) suggests that the failure of , say, 5.6×10^{-6} *M* ATP, to elicit contraction of isolated myofibrils, is caused by inadequate diffusion of ATP, which becomes a critical factor (even in isolated myofibrils) at these low concentrations. As Perry points out, these results show clearly that ATP is being used up inside the fibrils, and not merely bound, when producing contraction.

Bozler, using intact glycerinated fibers, was able to obtain contraction in very low concentrations of ATP ($\sim 10^{-6}$ *M*), providing that 20×10^{-3} *M* CP was also present. The tension took 10–20 minutes to develop, presumably a measure of the time taken for the low nucleotide concentration outside to saturate the fiber. The minimum amount of nucleotide required for maximum tension was found to be $2\text{–}4 \times 10^{-7}$ mole ATP per gram of muscle. This corresponds to one mole of ATP for every 200,000–400,000 gm. of myosin. Thus it seems probable that one nucleotide molecule becomes bound on each enzyme site (on each cross bridge in the model) and the creatine phosphate enables them to be constantly rephosphorylated, perhaps even *in situ*. The CP concentration is sufficiently high for diffusion of CP not to be a limiting factor.

An important (and slightly disappointing) observation made by Perry is that the ADP-CP induced contraction of myofibrils requires the presence of *added* ADP, i.e., the bound ADP of the fibrils (which we have seen is probably bound to the actin) is not available for this process, and indeed does not on its own cause phosphate to be liberated by the muscle ATPase in the presence of creatine phosphate.

The extent to which *in situ* rephosphorylation of bound ADP occurs during normal muscular activity is not easy to gauge, but Kuby *et al.* (1954b) have shown that purified CPase is an enzyme with an extremely high activity. Thus in the reaction

$$\text{ADP} + \text{CP} \rightleftharpoons \text{ATP} + \text{C}$$

the rate of the forward reaction can be 120,000 moles per minute per 100,000 gm. enzyme, that of the back reaction 20,000 moles per minute.

These authors calculate that in muscle, *all* the ATP could be resynthesized in 30 milliseconds. If this is the case, and if bound ADP is rephosphorylated at a similar rate, then the reaction would be fast enough to restore the terminal phosphate to the bound ADP molecules while a twitch was still in progress; in fact, the CP concentration might affect the rate of energy release during a contraction, if replacement of bound ADP by fresh ATP was a slower process.

d. Substrate inhibition. The manner in which overoptimal concentrations of ATP cause inhibition of ATPase activity and relaxation of fiber models is a matter of controversy. The effect is obtained only in systems containing actin and myosin, and does not occur with pure ATPase. Perry (1956) and Perry and Grey (1956) have described impressive evidence that the important factor is the ATP : Mg ratio, inhibition setting in over a substantial range of absolute ATP concentrations (2 to $10 \times 10^{-3} M$) when this ratio exceeds unity. These authors suggest that the true substrate for the myofibrillar ATPase may be Mg.ATP and that free ATP may act as a competitive inhibitor; thus an excess of ATP over Mg would lead to inhibition and relaxation. Substances which bind magnesium, like pyrophosphate, would also be able to produce relaxation, and it is suggested that the Marsh relaxing factor may act in a similar way.

Hasselbach (1957) and Geske *et al.* (1957), who have also studied the ATP : magnesium ratio effects, favor a slightly different interpretation of their results, which they consider are better explained if the concentration of free magnesium is the critical factor, the substrate for myofibrillar ATPase being free ATP.

The fact that the ATPase of pure myosin does not show substrate inhibition, and that only the magnesium-activated ATPase of myofibrils and of actomyosin gives the effect [there is no effect for instance, with myofibrils in the presence of calcium (Perry and Grey, 1956)] indicates that it is only the actin-controlled myosin ATPase which can be inhibited by excess of ATP. It is as though the acceleration of the ATPase by actin, which we have discussed in Section III, B, 2, a, was blocked by excess ATP—or lack of magnesium.

3. *Experiments on the Chemical Reactions Taking Place during Physiological Contraction in Vivo*

Introduction. Despite our lack of knowledge of the exact details of the reactions taking place during contraction and relaxation, it seemed quite self-evident that ATP must at least be split during *some* part of the contraction cycle. The current balance of evidence seemed very much in favor of the view that it is split during the contraction phase of the cycle, and we shall see later that this view is supported by the measurements of heat production at various phases of a twitch. At what point during the repeating cycle of operation of the individual cross bridges the reaction occurs is a question which the sliding filament model leaves open. Whatever the answer, however, this cycle repeats many times during the contraction phase of a twitch, and ATP should therefore be split during this phase.

It is therefore a somewhat embarrassing anticlimax to find that attempts to measure a change in the ATP or CP content of a muscle during a single twitch or a short tetanus at O° C. have so far produced a very negative result. The changes in ATP or CP content which have been measured are very much smaller than those required to supply energy for the work done and heat released by the muscle during the period of activity.

b. Observations. The most recent and thorough experiments reported are those by Fleckenstein *et al.* (1954) and by Mommaerts (1954, 1955). The procedure is to use two symmetrical muscles, one being quickly frozen in the resting state, the other during or after a contraction. Both muscles are then extracted and analyzed. Fleckenstein *et al.*, using the rectus abdominis (a slow muscle) of the frog, found (in experiments summarized in Table VI) that in 1–2 seconds tetani, produced by electric stimulation at 0° C., the change in the ATP level was less than ±0.04 μmole per gram muscle, that of the ADP level less than ±0.03 μmole per gram. The CP level appeared if anything to increase (mean increase on 20 pairs, +0.32±0.20 μmoles per gram) although this represented only a small change in a much larger basic level ($\sim$12μmoles per gram). On the other hand, the amount of work done by the muscle was about 125 gm.cm. per gram, and, taking normal values of shortening and activation heats, the total energy release must have been of the order of 300 gm.cm. per gram or more (i.e., efficiency not greater than 40% (Hill, 1949c)). If this energy was derived from the splitting of energy-rich phosphate bonds (liberating say 10,000 calories per mole), then the amount required would be 0.7 μmole per gram muscle. This is very much larger than the observed change in ATP and ADP. To make it easier to detect changes in the creatine phosphate content, the muscle was treated with 2, 4-dinitrophenol so as to lower the initial CP level to 0.6 μmole per gram. When this was done, the change in the CP level after a 1.3–3.5 seconds electrically stimulated tetanus was found to be less than ±0.1 μmole per gram, and the changes in ATP and ADP levels were still less than ±0.05 μmole and ±0.04 μmole per gram, respectively. The total energy released in this experiment was equivalent to about 0.6 μmole energy-rich phosphate per gram. In both the experiments small decreases in the "total organic phosphate less creatine phosphate" content were observed, and in the second experiment

TABLE VI (from Fleckenstein *et al.*, 1954)

A. CHANGES IN PHOSPHATE-CONTAINING COMPOUNDS AS A RESULT OF A SINGLE CONTRACTION AT 0° C. CAUSED BY IMMERSION FOR 45 SECONDS IN 0.765 % POTASSIUM CHLORIDE SOLUTION

	Fraction					
Notes	a Creatine phosphate (μmoles/gm.)	(a+b) Creatine phosphate + the inorganic phosphorus (μmoles P/gm.)	c Adenosine triphosphate (μmoles/gm.)	d Adenosine diphosphate (μmoles/gm.)	e An unknown fraction *Dritte* (μmoles P/gm.)	Work done by the muscle (gm.cm./gm.)
8 Pairs						
Test	—	18.5	1.80	1.44	2.10	136 ± 8
Control	5.99	18.0	1.85	1.53	2.59	—
4 Pairs						
Test	5.99	—	—	—	—	81 ± 5
Control	7.44	—	—	—	—	—
Mean difference	−1.45	−0.5	−0.05	−0.09	−0.49	—
Standard error of mean difference	±0.25	±0.5	±0.02	±0.08	±0.16	—

B. CHANGES IN PHOSPHATE-CONTAINING COMPOUNDS AS A RESULT OF A SINGLE CONTRACTION AT 20° C. OR 0° C. PRODUCED BY 2½ MINUTES IMMERSION IN ½ % CAFFEINE-RINGER SOLUTION

Notes	Fraction					Work done by the muscle (gm.cm./gm.)
	a Creatine phosphate (μmoles/gm.)	b Creatine phosphate + the inorganic phosphorus (μmoles P/gm.)	c Adenosine triphosphate (μmoles/gm.)	d Adenosine diphosphate (μmoles/gm.)	e An unknown fraction *Dritte* (μmoles P/gm.)	
20° C.						
6 Pairs						
Test	—	19.9	1.20	1.17	0.91	162 ± 23
Control	—	16.3	2.13	1.05	1.68	—
6 Pairs						
Test	0.57	—	—	—	—	154 ± 19
Control	6.68	—	—	—	—	—
Mean difference	−6.11	+3.62	−0.93	+0.12	−0.77	—
Standard error of mean difference	±0.37	±0.31	±0.12	±0.068	±0.26	—
0° C.						
6 Pairs						
Test	—	17.4	2.17	0.92	1.66	109 ± 15
Control	—	17.3	2.21	1.03	1.77	—
5 Pairs						
Test	2.65	—	—	—	—	82 ± 4.1
Control	6.36	—	—	—	—	—
Mean difference	—3.71	+0.1	−0.04	−0.11	−0.11	—
Standard error of mean difference	±0.60	±0.87	+0.09	±0.06	±0.26	—

C. CHANGES IN PHOSPHATE-CONTAINING COMPOUNDS AS A RESULT OF A SINGLE CONTRACTION AT 20° C. CAUSED BY 1.0–1.2 SECONDS ELECTRIC STIMULATION

Notes	Fraction a	(a + b)	c	d	e	f	g
	Creatine phosphate (μmoles/gm.)	Creatine phosphate + the inorganic phosphorus (μmoles P/gm.)	Adenosine triphosphate (μmoles/gm.)	Adenosine diphosphate (μmoles/gm.)	An unknown fraction (μmoles P/gm.)	Total organic phosphorus the creatine phosphate (μmoles P/gm.)	Work done by the muscle (gm.cm./gm.)
20° C.							
12 pairs							
Test[a]	11.25	24.93	2.77	0.87	1.14	15.36	190 ± 11.1
Control	13.32	25.70	2.77	0.91	1.20	16.53	(47.7 ± 4.4)[b]
Mean difference	−2.07	−0.77	0.00	−0.04	−0.06	−1.17	—
Standard error of mean difference	±0.38	±0.51	±0.053	±0.049	±0.14	±0.33	—
0° C.							
20 pairs							
Test	11.46	22.21	2.82	0.71	0.96	15.50	125.3 ± 20.8
Control	11.14	21.96	2.80	0.70	0.93	15.70	0 ± 0.0
Mean difference	+0.32	+0.25	+0.02	+0.01	+0.03	−0.20	—
Standard error of mean difference	±0.20	±0.39	±0.040	±0.032	±0.073	±0.27	—

[a] In this series the total acid-soluble phosphorus in the test muscles was less than in the control muscles. Fraction (a + b) represented 61.9 % of the acid-soluble phosphorus in the test series and 60.9 % in the control series. For further details see Fleckenstein *et al.* (1954).

[b] The control muscles contracted somewhat on freezing. This did not occur with control muscles at 0° C.

D. CHANGES IN PHOSPHATE-CONTAINING COMPOUNDS AS A RESULT OF A SINGLE CONTRACTION AT 0° C. CAUSED BY 1.3–3.5 SECONDS ELECTRIC STIMULATION AFTER 20 MINUTES' PRETREATMENT WITH 5×10^{-4} *M* 2, DINITROPHENOL

Notes	Fraction						
	a Creatine phosphate (μmoles/gm.)	(a + b) Creatine phosphate + the inorganic phosphorus (μmoles P/gm.)	c Adenosine triphosphate (μmoles/gm.)	d Adenosine diphosphate (μmoles/gm.)	e An unknown fraction (μmoles P/gm.)	f Total organic phosphorus the creatine phosphate (μmoles P/gm.)	Work done by the muscle (gm.cm./gm.)
12 Pairs							
Test	0.58	21.18	1.76	0.91	0.31	12.44	109.8 ± 4.7
Control	0.62	20.77	1.75	0.87	0.29	13.14	0 ± 0.0
Mean difference	−0.04	+0.41	+0.01	+0.04	0.02	−0.70	—
Standard error of mean difference	±0.100	±0.45	±0.048	±0.040	±0.073	±0.306	—

only, a small increase in the total inorganic phosphate occurred during the tetanus. However, in each case the amounts involved were of the same order of magnitude as the probable error in the measurements, and in the normal muscle at 0° C., the decrease in organic phosphate ($0.2 \pm 0.27\ \mu$mole per gram) was less than one-third the amount required to supply the energy.

After short tetani at 20° C., and after KCl- or caffeine-induced contraction at 0° C., the ATP and ADP levels were found still to be unchanged, but the CP level fell considerably; it was apparent that ATP could have been resynthesized at the expense of CP. In a caffein-induced contraction at 20° C., almost all the CP disappeared, and the ATP level fell also—without, curiously enough, much change in the ADP level.

Mommaerts studied the changes occurring during single twitches of the leg muscles of the tortoise. He, too, found only very small changes in the ATP, ADP, and CP levels, which he was able to measure at different phases of the twitch. The variations observed—$\pm 0.2\ \mu$mole for ATP and CP, and $\pm 0.1\ \mu$mole for ADP—seemed to be entirely random, and no systematic changes greater than $0.05\ \mu$mole were considered to have taken place. The total energy released in these experiments was equivalent to the breakdown of about $0.5\ \mu$mole of energy-rich phosphate.

c. Discussion. It is not considered likely by these authors that resynthesis of ATP or of CP through any known pathway could have taken place with sufficient speed to account for the findings. Rephosphorylation of ADP by CP cannot explain the results for the CP levels do not change sufficiently. There seem to be only a limited number of ways out of this paradoxical situation.

One way might be (as Fleckenstein *et al.* pointed out) to suppose that there is a hitherto undetected energy-rich phosphate in muscle which is the real substrate for the enzymatic reaction involved in contraction, that the concentration of this substance is normally maintained by transphosphorylation from ATP, and that a limited amount of work can be done by the muscle using this substance as an immediate energy source, before any changes in ATP or CP levels occur. Alternatively, the existence of an unknown phosphate which could rapidly rephosphorylate ADP (in the same way that CP does) might be suggested. But as we have seen, the liberation of inorganic phosphate and the disappearance of organic phosphate do not really occur to a large enough extent to give much support to this type of theory.

Alternatively, it might be supposed that the energy transfer to the protein structure has already taken place before the contraction starts, so that no further breakdown of ATP takes place during contraction. One would rather expect, then, that the energy transferable in this way would be limited to that associated with one phosphate group per enzyme site. Such an interpretation does not fit in very well with the figures for the number of enzyme sites believed to be present in the muscle, and the quantity of energy released without apparent fission of ATP. The number of sites was shown earlier to be in the range 4 to 8×10^{16} per gram of muscle, whereas the number of energy-rich phosphate bands broken in these contractions is about 42×10^{16} per gram of muscle. Thus five to ten transfers must take place at each site to account for the observed energy release, and not just a single transfer. It is difficult to imagine that these all take place beforehand.

If fresh muscle contains ADP bound to actin in the same amounts that are found in purified actin, then the quantity involved would be about 0.4 μmole per gram. The amount of ADP found in the experiments we have been discussing was two or three times as large as this value. Thus both the control and the experimental muscles contained 0.4–0.8 μmole of ADP not bound to actin. It would clearly be desirable to exclude the possibility that this ADP was formed in the control muscle at some point during the freezing and extracting procedures, in sufficient amounts to match the quantity of ADP in the experimental muscle which had contracted.

No doubt further experiments will solve this present mystery. All the other evidence fits in very well with the view that ATP is dephosphorylated during the rising phase of a muscle twitch. It will be interesting to see just at what point the experiments or the interpretations have gone wrong.

IV. The Mechanical and Thermal Properties of Striated Muscle

We have already referred briefly to some of the physiological properties of muscle; we shall now return to this topic and consider the properties in greater detail, in the light of the structural and biochemical discussions, seeing, wherever possible, whether they give information about the basic molecular mechanisms involved in contraction. This can best be done by discussing separately the behavior of resting muscle, the behavior of active muscle, and the behavior of muscle during the transition between the two states; that is, during activation and during relaxation. Some of the phenomena may seem esoteric, and unimportant to the function of muscles; but it is often these which give the most interesting insights into the nature of the process.

A. *Resting Muscle*

Muscle in the resting state is readily extensible, as indeed it must

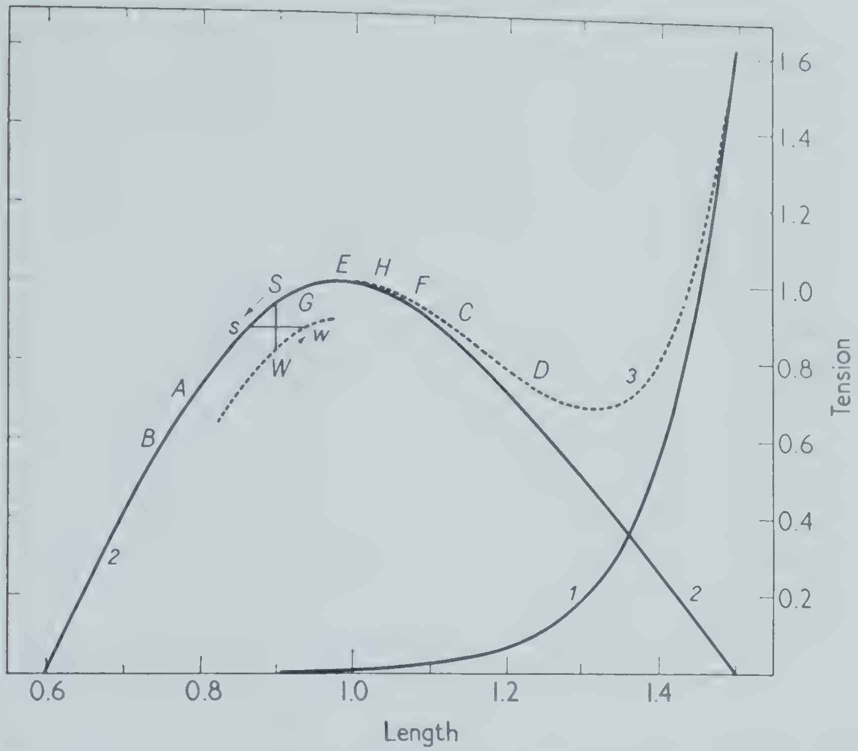

FIG. 30. Diagram of tension-length relations of sartorius muscle of frog or toad; *1*, at rest, passively stretched; *2*, extra force developed during maximal tetanus; *3*, dotted line, total force in maximal tetanus, sum of *1* and *2*. The length is given as a fraction or multiple, of the standard length in the body; the tension of the maximum force developed. From Hill (1953a). (Reproduced by kind permission of the Royal Society.)

be for obvious reasons if it is to form part of a mechanically efficient system. If it has previously shortened to below rest length it may be re-extended by a very small force, and it will not exert appreciable tension until the resting length of the muscle is considerably exceeded Fig. 30) (Hill, 1953a). This plastic behavior of the contractile substance s easy to understand in terms of the sliding filament model, in which at rest, the two sets of filaments are not linked together and can be drawn past each other by forces sufficient to overcome the viscous resistance (or its equivalent at this structural level).

When a resting muscle is stretched beyond its normal length in the body, the tension in it increases slowly at first and then more rapidly, n the manner illustrated in Fig. 30. In stretches of 10 or 20% of the muscle length, the tensions are very small compared with those needed to stretch the series elastic component only a few per cent of the muscle length. The component (or components) responsible for these resting tensions are called the parallel elastic components. To them, the sarcolemma may contribute, although the work of Casella (1950) suggests that the contribution does not become appreciable until extensions of more than

20 or 30% occur. These components could arise in two other rather less obvious ways.

In the first place, as the volume of the muscle fiber remains constant during a stretch, the cross-sectional area must decrease, and hence the side spacing between the filaments must decrease, for electron micrographs show that the array of filaments at rest length occupies virtually the whole area of the fibers; in fact, low-angle X-ray diffraction studies (H. E. Huxley, 1952) showed such a decrease in transverse spacings during stretch. If a force of repulsion exists between the filaments (as some such force must do in order to maintain them in a regular array), then work must be done in order to move them closer together. The necessary force will appear as a "parallel" elastic resistance to stretch.

Secondly, good evidence exists for the presence of a connection between the ends of the secondary filaments, across the H-zone. Resting muscles under zero tension shorten slightly from their rest length (R.L) in the body, to a so-called "equilibrium length" (A. Szent-Györgyi, 1951), about 85% R.L. In such slightly shortened muscles, the ends of the actin filaments seem to have just come together in the center of the H-zone (Hanson and Huxley, 1955). And if the array of primary filaments (myosin) is dissolved out from a muscle fibril which was held stretched during glycerination, so that it "set" in the stretched state, then the "ghost" fibril shortens down if it is not restrained, and the H-zone closes up. Such a connection—described as the S-filaments (H. E. Huxley and Hanson, 1954)—would behave as a parallel elastic component.

The heat rate of resting muscle is very low compared to the maximum rate of energy release during activity. Thus, for frog sartorius at 18° C., resting heat amounts to 2×10^{-4} calories per gram per minute (Wilkie, 1954), while maximal activity releases about 4 calories per gram per minute. Even if the resting heat were entirely due to the breakdown of ATP by myosin, then only approximately one molecule of ATP would need to be split every 200–300 seconds at each active site. It is apparent that the ATPase activity of the myosin is very effectively inhibited indeed when the muscle is in the resting state.

B. Active Muscle

1. The Force-Velocity Relation

When a striated muscle is in the active state, it can exert a maximum tension of 1 or 2 kg. per square centimeter of its cross section. Higher values than this have appeared in the literature [e.g., Haxton (1944) gives a value of 3.9 kg.cm.^{2} for human muscle] but the more usual figures—for instance, those apparent in most of A. V. Hill's papers on frog sartorius muscle—lie around 1.5 kg.c.m.^{2}, and we shall use this figure in any calculations which we carry out. Maximum tension is obtained only when the contraction is an isometric one, i.e., when the muscle is not allowed to shorten. If the muscle is shortening, then the tension it exerts is less than the maximum tension, and the greater the shortening velocity, the lower the tension will be. One might at first suspect this to be due to some sort of internal viscous resistance. This does not seem to be the case however, at least not to an important extent. Viscous resistances increase with velocity, and if the observed decrease in tension was brought about in this way, one would expect larger amounts of heat to be generated in the system, for a given distance of shortening, at higher velocities of shortening. This does not happen in practice. When a muscle lifts a smaller load, and so does less work while shortening the same distance with a higher velocity, the heat liberated is almost the same as when much larger loads are being raised (Hill, 1949c). This observation not only rules out internal viscosity as the factor responsible for the shape of the force-velocity diagram, but it also shows that the contractile machinery can liberate varying amounts of energy when it changes its length by a constant amount. As the total energy release must be the sum of the energies released by a large number of individual molecular events, it follows that the number of these events, or reactions, depends not so much on the initial and final configuration of the muscle as on the tension exerted by it during the transition between the two configurations. If we think of tension as being generated at a number of sites acting in parallel, then the idea immediately arises that perhaps ATP can be split, and energy liberated, only at sites which are generating tension. In this way, a controlling link could be established between the external work being done and the energy being released by the muscle.

We have already discussed how the force-velocity relations may be described by the Hill equation (Section I, D):

$$(P+a)\,v = (P_0-P)\,b$$

and we have drawn attention to the significance of the fact that the equation applies over the whole range of physiological shortening, providing the value of P_0 appropriate to the length in question is used (Abbott and Wilkie, 1953); the values of a and b remain constant. It is reasonable to suppose that the equation expresses some characteristic property of the fundamental molecular processes involved in contraction, and its simplicity suggests that there ought to be some very simple way of visualizing these processes.

More remarkable still, however, is the fact that the constant a in the force-velocity equation turns out to be equal to the heat liberated by the muscle per unit distance of shortening (Hill, 1938). That these two quantities, one determined by investigations of the dynamical behavior of a muscle ($a_{\text{dynamical}}$), the other by heat measurements on it (a_{thermal}), should be the same, and should remain the same at different muscle lengths (Abbott, 1951), seems too improbable to be purely coincidental. It suggests that a very close and necessary relation exists between them.

2. *The Relation between Load and Rate of Energy Release*

Inspecting the Hill equation again, we see that the left-hand side $(P+a)\,v$ gives us the external work rate plus the shortening heat rate. If for a moment we ignore maintenance heat, the equation tells us that the system behaves as though the total rate of energy production was $(P_0-P)\ b$, provided that $a_{\text{dynamical}}$ and a_{thermal} are identical. This particular relationship is a very interesting one, for it shows, as was pointed out by Hill (1938), that if we regard P_0 and P as being in some way proportional to the number of "linkages" involved in generating tension, then (P_0-P) is a measure of the number of "free" linkages in a muscle which can develop a maximum tension P_0 and which is shortening at such a velocity that its actual tension is only P. The rate of energy release is then proportional to the number of *free* linkages. This may seem somewhat parodoxical in the light of our earlier remarks about energy being released only at linkages which were generating tension; but it makes sense if the rate-limiting step in the contractile reaction is the reactivation of free, inactive linkages to ones which are generating tension. The total rate at which this occurs will be proportional to the number of such inactive linkages (i.e., proportional to (P_0-P)), and hence the rate of energy release will be proportional to this quantity too.

In the sliding filament structure, the cross bridges correspond to the "linkages" whose tensions add up in parallel. Each time a cycle of operation has taken place at a cross bridge, the bridge becomes free to commence a new cycle and generate tension again. If the muscle were exerting a large tension, then most cross bridges would be active and the "pool" of inactive bridges waiting to commence a new cycle would be small; if the reactivation process is slow compared with the other steps in the cycle of operation, then the rate of energy production would necessarily be low. On the other hand, a small tension would imply a large pool of bridges available for reactivation, and a high rate of energy production.

It is rather like having a team of men pulling in a long rope. If the rope is being drawn in rather quickly, then at any instant several men will always be in process of shifting their grip on it after completing one cycle of pulling, and the tension will be less than the maximum which could be exerted by all the men simultaneously. The total rate at which work is being done can be ascertained by counting the number of men *not* pulling at any moment (say, 7), and observing how long it takes them to change their grip (say it was 1 second). Then the total rate of working must be 7 man-cycles per second, for there must be a steady flow of seven men per second into the pool of those who have just completed a cycle of pulling.

This rather primitive explanation of the Hill equation has, it should be noted, some similarities to the more sophisticated one given by A. F. Huxley (1957); important differences do exist, however. The present remarks are intended just to give a rough, but direct, physical picture of how the particular mathematical form of the relation between load and rate of working could arise.

3. *The Relation between Length and Tension*

The shape of the length-tension diagram deserves some comment at this point. The curve showing additional tension (above resting tension), developed during activity, has been given in Fig. 22, and we have mentioned how A. F. Huxley and Niedergerke (1954) have pointed out that the decreasing tension beyond rest length might be explained in terms of the decreased extent of overlap in the sliding filament model.

More difficult to explain, however, is the decrease in tension below rest length. The length of the overlap region remains about constant until the Z-lines have been drawn into contact with the ends of the A-bands; perhaps a small decrease in the number of available cross bridges near the centre of the A-band might result from the "piling up" of the secondary filaments in that region, but hardly enough, one would imagine, to produce such a large decrease in tension. Nor does it seem possible that this "piling up" of secondary filaments could provide a resistance to shortening which would appreciably reduce the externally measurable tension, as suggested by A. F. Huxley (1957). If it did so, then either extra heat would be generated in it during shortening, or else the energy stored in it during contraction would be degraded into heat during relaxation. Neither of these effects is observed. The heat of shortening remains constant over the whole range of lengths (Abbott, 1951), and no heat is given out during relaxation, provided that the potential energy stored in the *load* is not dissipated as heat in the muscle (Hill, 1949b).

A different explanation may perhaps be sought along the following lines: As a muscle fiber shortens, its volume remains constant, and therefore its diameter increases. As a result, the filaments may move further apart. This might decrease the probability of a cross bridge becoming attached and generating tension; or it might cause the bridges which were attached to be oriented more nearly at right angles to the filaments, and so to exert a smaller component of tension in an axial direction.

The decrease in tension at shorter lengths is not accompanied by any change in the heat of shortening. This rather suggests that the heat of shortening is not produced directly by the reaction which generates tension.

4. *The Relation between Tension and Number of Cross Linkages*

From the X-ray and electron microscope observations, it can be calculated that the number of cross linkages in a volume of muscle one-half sarcomere in length and 1 cm.^{2} in cross section is approximately 6.5×10^{12}. If this square centimeter of muscle is generating a tension of 1.5 kg. and has a heat of shortening of 350 gm.cm. per centimeter, then the extra energy released by the muscle when it shortens a distance of 100 A. is 1.81 ergs; and hence the energy released at each cross bridge is about 2.8×10^{-13} ergs. If the energy liberated by the splitting of one gram molecule of ATP is 10,000 calories, then the energy from a single molecule of ATP is 2.8×10^{-13} ergs. Thus, on this model, one molecule of ATP would be broken down at each of the cross bridges when the secondary filaments moved along past the primary filaments by a distance of 100 A.

This is a very reasonable value for what we shall refer to as the "step distance." The separation between points on the secondary filaments to which the bridges appear to be attached is about 133 A., and if these were the only points at which attachment could take place, then clearly the secondary filaments would have to move along at last this distance each time one cycle of operation of a cross bridge takes place. If the possible attachment points were 54 A. apart on the actin filaments, as suggested by the Selby-Bear actin structure (1956), then one would expect the maximum tension generated by the muscle to be about 3.1 kg./cm.^{2}. It is a puzzling thing that the tensions reported for glycerinated rabbit psoas muscle (4–5 kg./cm.^{2}, Weber and Portzehl, 1952) are so much larger than those usually found in live frog sartorius muscle; the tensions are so large that they will cause fibers around rest length to tear themselves to pieces (Portzehl, 1957); isolated fibrils, too, irrigated with physiological concentrations of ATP, will break themselves if they are restrained from shortening (Hanson and H. E. Huxley, unpublished observations). One wonders whether, in a living muscle, the bridges are never able all to generate tension simultaneously.

5. *The Relation between the Maximum Rate of Energy Release and Number of Cross Linkages*

Energy is liberated at a maximum rate by the muscle when it is shortening with zero load. For a frog muscle at 0° C., shortening at a rate of about 2 muscle lengths per second, it is easy to show that about 20 molecules of ATP must be then broken down at each site per second (neglecting maintenance heat); this rate is comparable with the splitting rate of ATP by pure myosin under optimal conditions. When the muscle is lifting a load, the rate of energy release is reduced; with half maximum load it would be equivalent to 10 molecules per site per second. It will be apparent that these are quite low frequencies.

6. *Maintenance Heat*

When an active muscle is not allowed to shorten at all, so that the "heat of shortening" is zero, and no external work is done either, the liberation of heat does not cease altogether, but continues at a rather low rate—about 128 gm.cm. per gram per second in frog sartorius in tetanus at 0° C. (Hill, 1938), i.e., about one-sixth the maximum rate during free shortening. The "heat of shortening" is the extra heat over and above this baseline, which is liberated when the muscle is allowed to shorten. As has been pointed out by A. F. Huxley (1957), this convenient but arbitrary division of the heat into two parts should not lead us to assume without further proof that the distinction is a real one; the fact that the extra heat is proportional to the distance shortened does not prove that the heat is evolved from different processes.

However, it is interesting to note that the maintenance heat evolved during a tetanus varies with the muscle length, being smaller at the shorter lengths, where less tension is exerted; the maintenance heat is roughly proportional to the isometric tension at the length in question (Aubert, 1956). This is in clear contrast to the constancy of heat of shortening over a wide range of muscle lengths; it rather suggests that the maintenance heat is directly related to the events which produce tension, and that the shortening heat is a *result* of the shortening process, rather than an immediate concomitant of the tension-generating events themselves.

In a single twitch, there seems to be a certain basic amount of heat produced too, on top of which heat of shortening is added; if no shortening takes place [e.g., in the case of a fully preshortened muscle, or a muscle extensively stretched by a load which it cannot lift (Hill, 1950d)], then this heat (called by Hill "activation heat") appears alone. In the case of frog sartorius muscle at 0° C. it amounts to 80 gm.cm. per gram (Hill, 1948), and in toad sartorius, 30–40 gm.cm. per gram. This would correspond to the breakdown of one or two molecules of ATP at each site during the twitch.

7. *Behavior of Muscles Stretched While in the Active State*

a. Dynamic behavior. If a load greater than the maximum isometric tension is applied to a contracting muscle, then the muscle lengthens. The velocity of lengthening is very much less than would be obtained if the Hill equation could be applied to the process (Katz, 1939). This is a rather unexpected result and attention has been redirected to it by A. F. Huxley's (1957) considerations in terms of his particular mechanism; one might have supposed that the processes involved in lengthening were exactly the reverse of those involved in shortening and that therefore no discontinuity in the force-velocity diagram should arise at $v=0$. The experimental observations therefore suggest that the contractile elements in a lengthening muscle are in a state which is structurally different from that of the same elements during shortening. In the sliding filament model, such a difference can occur if the cross bridges are pulled beyond their normal range of operation by becoming attached to the secondary filaments which are moving, as it were, in the wrong direction. (It is this process which A. F. Huxley has considered, in terms somewhat different from the following.)

The maximum tension which can now be exerted (i.e., during lengthening) will depend on the strength of the attachment between the cross bridges and the secondary filaments, and the velocity of lengthening will depend on the rate at which cross links detach under the stress which is being applied to them when they are pulled out of their normal positions. This is a different process altogether from the one we suppose takes place during contraction (when detachment takes place automatically), and it is therefore likely to lead to different force-velocity relations.

When the applied tension is increased, a point is reached (at $1.8P_0$ in frog sartorius muscle (Katz, 1939)) at which the muscle begins to lengthen very rapidly, to "give"; presumably that tension is sufficient to break the attachments very rapidly. At velocities higher than this, the force exerted seems to depend on the distance stretched and not on the velocity of stretching (Abbott and Aubert, 1952). Moreover, after lengthening is over, the tension decays to a value which is higher than the normal isometric tension at that length (see Fig. 31). If we are to explain tension exerted during a stretch on the basis of abnormal displacement of the cross bridges, then we are forced to conclude that some of the bridges may remain attached, in the displaced configuration, for a substantial time after the stretch is over, and only return to their normal position if the muscle is allowed to relax and is then restimulated (when the normal isometric tension is developed). It is as though the cross bridges were "locked-on" at small displacements, but would detach rapidly at larger displacements, when the stress exceeded a certain value. We shall

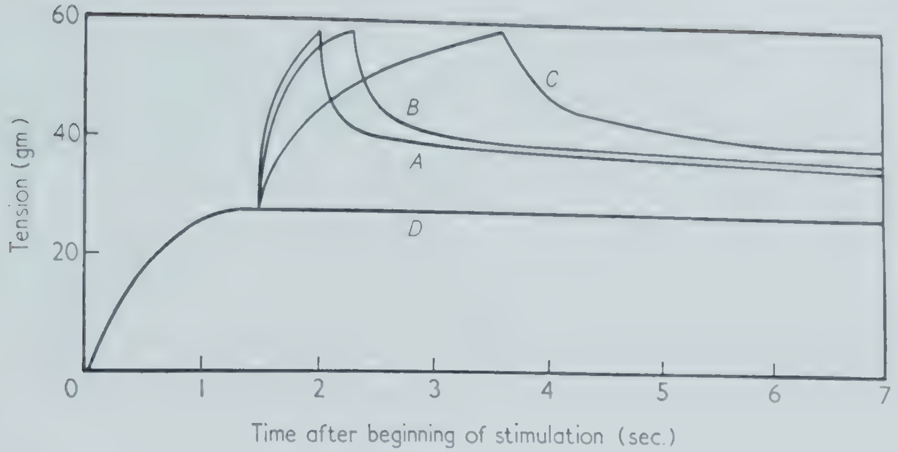

FIG. 31. Tension during and after stretch of toad sartorius muscle at 0° C. (standard length, 28 mm.; weight, 134 mg.). Stretch from 21.5 to 25.5 mm. began after 1.5 seconds of stimulation: *curve* A at 8 mm. per second; *B* at 5 mm. per second; C, at 1.9 mm. per second; D, isometric at final length 25.5. Stimulus frequency, 3 per second. From Abbott and Aubert (1952). (By courtesy of the *Journal of Physiology*.)

bear this in mind when we discuss somewhat related anomalies which occur during shortening.

b. Thermal behavior. When an active muscle is slowly stretched, some very extraordinary effects are observed on the heat production by it (Abbott, *et al.*, 1951; Abbott and Aubert, 1951). Instead of the work done on the muscle by the load appearing as heat, a considerable part, or even all of it, disappears. By this, we mean that assuming the maintenance heat has remained constant, the additional heat output by the muscle as a result of stretch is less than the work done on it. Thus during a 3½-mm. stretch (in 1 second) of a frog sartorius muscle in tetanus at 0° C., between 50 and 60% of the work done on the muscle disappeared, and a 3½-mm. stretch in one-sixth of a second early in a twitch led to the disappearance of about 40% of the work done.

These velocities of stretch are higher than the velocity at which the muscle "gives." At lower velocities of stretch (up to about 0.5 mm. per second), obtained with loads between $1.2\,P_0$ and $1.5\,P_0$, all the work done on the muscle was found to disappear (Abbott and Aubert, 1951). Moreover, the heat liberated by the muscle became less than the isometric rate averaged over the range of lengths through which the muscle was stretched. The muscle exhibited, apparently, a negative heat of lengthening, which varied from about 400 gm.cm. per cubic centimeter at the higher speeds of shortening ($\sim$ 0.4 mm. per second) up to 2000 gm.cm. per cubic centimeter at the very lowest speeds of lengthening ($\sim$ 0.15 mm. per second). However, in no case did the work done on the muscle exceed

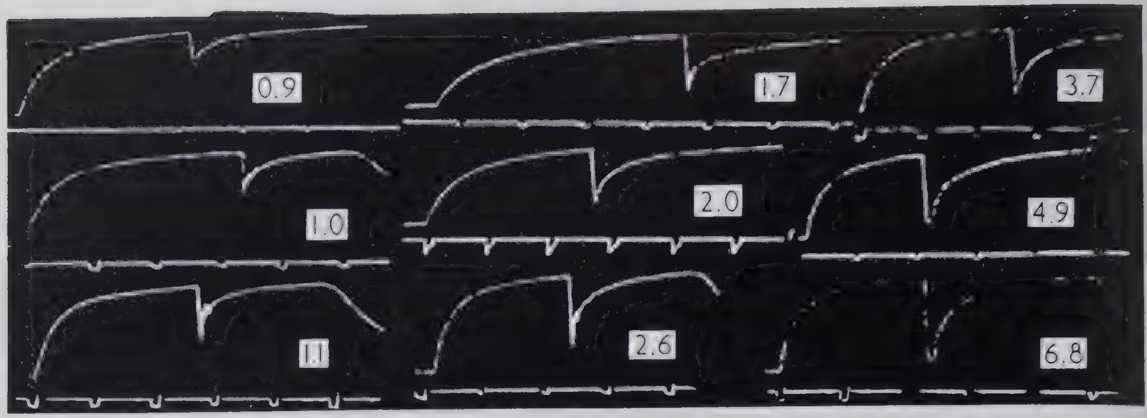

FIG. 32. Ordinate, tension; abscissa, time. Isometric tetanus of biceps cruris of tortoise (65 mm. long, room temperature), with quick release during the stimulus. The numbers on the curves represent the extent, in millimeters, of quick release. Time marks, seconds. From Hill (1926a). (By kind permission of the Royal Society.)

the heat output by it; thus the muscle never gained an internal energy, even during the slowest stretches.

The complete disappearance of the work done on the muscle is a most remarkable phenomenon; it is not stored as elastic energy, for it does not reappear as heat during relaxation. It seems that either it really must be transformed into chemical energy, or else the stretching process must greatly reduce the normal maintenance heat and allow the work done on the muscle partly to take its place. If the former is the case, then the chemical reaction occurring at the cross bridges appears more remarkable than ever, for when the machinery is driven backward, the energy-rich compound which is split during shortening must be resynthesized. Alternatively, we have already noted that some evidence (the persistence of extra tension after stretch) exists that the cross bridges tend not to detach themselves when a muscle is stretched; this might account for a decrease in maintenance heat. It would be interesting to know whether this decrease persists after the stretch is over.

8. *The Series Elastic Component*

If an active muscle, held at a certain length, is suddenly released so that it can shorten by a few per cent of its length, the tension exerted by it falls abruptly and then redevelops again at the shorter length, in the manner illustrated in Fig. 32 (Gasser and Hill, 1924; Hill, 1926a, b). This behavior has been interpreted in terms of the presence of an elastic component in series with the contractile component of the muscle. When the muscle is exerting tension, this series elastic component is stretched, the amount of stretch under the full isometric tension being about 3–4% of the muscle length l_0 (Hill, 1950b), or, according to Jewell and Wilkie

(1958), some 2% of l_0. If the muscle is rapidly released, the contractile component, whose force-velocity characteristics are described by the Hill equation, will not be able to shorten fast enough to prevent the series elastic component, which is virtually undamped, from shortening too. The tension will therefore drop, falling to zero if the extent and speed of release allow the series elastic component to shorten completely. The tension will then build up again as the contractile component "catches up" and restretches the series elastic component.

The structural features of striated muscle which give rise to the series elastic component are very difficult to determine. The amount of stretch under maximum load is equivalent to about 500–1000 A. per sarcomere, or even less, and it is therefore not practicable to locate by optical means in which of the bands it occurs—if indeed it occurs in the contractile elements at all. Some of it probably occurs in the tendon connections at the ends of the muscle. Some of it could simply be elastic stretch of the protein filaments, but as Hill (1953a) has shown, even with an elastic modulus similar to that of rubber, they would account for only about one-tenth of the extension. The series elastic effect might also arise in a rather different way, once pointed out to me by A. F. Huxley; the elements which produce shortening might be able to shorten a small distance very rapidly in a release, and then have to wait to begin a new cycle of operation. Thus the tension would drop and then recover. A 100 A. step distance (Section IV, B, 4) would account for a shortening of the muscle by 1% of l_0 in this way. Hill (1953b) has shown that the heat given out during such releases is three or four times as large as the normal heat of shortening over the same distance. It is tempting to suggest that this occurs because, at the beginning of the release, at the higher tension, a large number of the cross bridges have begun a cycle of operation, which they have to complete, even though the muscle then shortens against a smaller load than they would normally operate against; the excess energy would appear as heat.

Again, any tension exerted by the cross bridges must, if they are at all obliquely oriented, have a component which tends to move the primary filaments closer together. This would allow the bridges to tilt farther and hence cause the muscle to stretch, when tension was applied.

The presence of the series elastic component has important consequences for the manner in which muscle tension develops and decays at the beginning and end of a period of activity; these will be discussed later. It also plays a part in some curious features of the behavior of muscles associated with rather rapid changes in length, which we will discuss now.

9. *Tension Development by Muscle after Special Types of Stretch and Release*

a. After abrupt changes of length and load. Gasser and Hill (1924) found that if an active muscle was very rapidly stretched, the tension in it rose abruptly and then fell again before rising to the value appropriate to its new length. The form of the latter part of the curve was very similar to that of the initial development of tension at that length. The initial sharp rise and fall of tension could represent stretch of the series elastic element, followed by the extension of the contractile component to its new length. The delay in the development of the final tension implies that immediately after a very rapid increase in length, the contractile component is unable to sustain its normal tension.

An analogous phenomenon has been observed by Katz (1939), who has also described a somewhat related effect during shortening. If a muscle, initially shortening against a small load, is prevented from shortening any further, the tension in it will build up as the series elastic component is stretched by the contractile component. If a load equal to the isometric tension is imposed on the muscle during the build-up of tension in it, the muscle re-extends again, and then shortens to the length appropriate to the tension imposed. The amount of extension observed was much larger than would be accounted for in terms of an elastic effect and indicated that the contractile component, when it is shortening and exerting less than its isometric tension, cannot immediately exert isometric tension if the shortening is arrested. It has been pointed out by A. F. Huxley (1957) that this latter phenomenon finds a ready explanation in terms of the sliding filament model; and perhaps the same type of explanation can be applied to the first phenomena described: If tension is generated by cross linkages, acting in parallel, between primary and secondary filaments, and if a deficit of tension below the isometric level during shortening (or lengthening) occurs essentially because the full number of linkages do not have time to become attached as the secondary filaments move past them, then when this movement is arrested, a finite time will elapse before the full complement of cross linkages can become established.

b. After moderate-speed stretches and releases. So far, departures of the observed tensions from those appropriate to a particular length or shortening velocity have occurred when the system is obviously not in a steady state and when previous conditions very naturally would still be affecting it. However, there is another class of phenomena, in which previous changes produce an effect which persists, and which are much more difficult to account for at present.

After the end of a moderate-speed stretch of an active muscle, tension

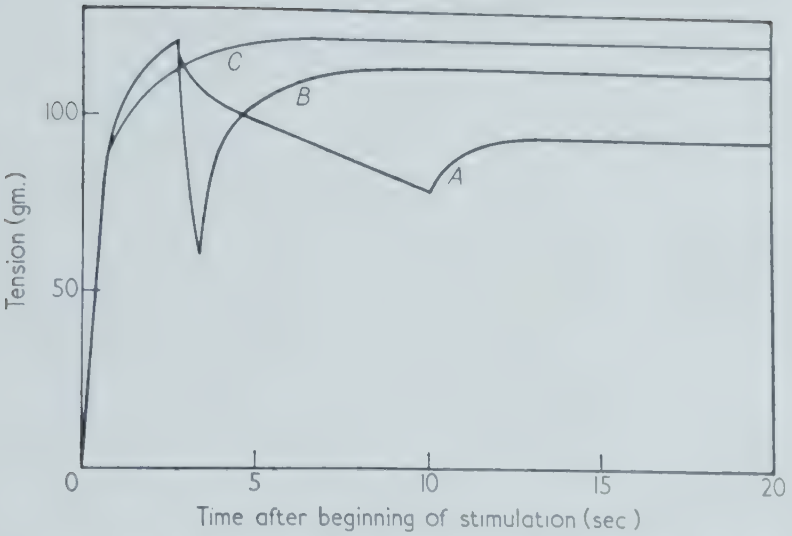

FIG. 33. Recovery of tension after shortening at different speeds. Dogfish jaw muscle, 0° C. Standard length 33 mm., weight 540 mg. Stimulus frequency, 5 per second. Shortening 42–37 mm. *Curve A* at 0.5 mm. per second; *B*, at 8 mm. per second; *C*, isometric, 37 mm. From Abbott and Aubert (1952). (By courtesy of the *Journal of Physiology*.)

decays to a value substantially in excess of the isometric tension at the length (Abbott and Aubert, 1952) and remains at that value for the rest of the tetanus. If the stimulus is stopped, and then restarted, normal tension is developed, so no irreversible change in the muscle substance has occurred. The stretch has produced a change which persists as long as the muscle remains active, and we have already mentioned the possibility that this might be due to cross links being moved during stretch to a position at which they could not detach from the secondary filaments.

During shortening too, a similar anomalous phenomenon has been described (Abbott and Aubert, 1952). After a rapid release, the fall in tension in the muscle reverses and the normal isometric tension develops again, in a manner which has been fairly satisfactorily explained in terms of the series elastic component. However, if the muscle is released the same distance, but more slowly, the recovery of tension is incomplete, and the degree of incompleteness increases as the speed of release dcreases, as shown in Fig. 33. Again, if the tetanus is interrupted, the normal isometric tension can be developed when it is restimulated. It appears that the contractile system can under these circumstances be in a steady state, and not be shortening, yet be exerting a tension which is lower than isometric. Again this seems to indicate that the pattern of cross linkages can sometimes depend on the previous history of the muscle. This point is an interesting one, but there is not time to pursue it further here.

C. The Transition between Rest and Activity: the Onset of Activity

1. Mechanical Effects

a. Introduction. When a muscle is stimulated, a period of delay intervenes between the passage of the action potential along the muscle membrane and the attainment of a state of full activity by the contractile substance. This delay is still observed even if the membrane is depolarized all over more or less simultaneously, so that no complications are introduced by the finite time of spread of activity along the length of the muscle. The extent of the delay depends on what criteria we use to measure the degree of activity of the contractile substance, and it varies considerably from one type of muscle to another. Thus, for example, it is longer in toad sartorius than in frog sartorius, and longer still in the iliofibularis of tortoises.

The simplest way of investigating the degree of activity of a muscle during a twitch is to measure the time course of the isometric tension development. Tension can first be detected, in frog sartorius muscle at 0° C., between 10 and 15 milliseconds (msec.) after stimulation (Hill, 1951b) and it reaches a maximum about 200 msec. later. In toad sartorius, isometric tension can first be detected after about 20 msec. (Abbott and Ritchie, 1951) and the maximum is reached in about 700 msec. In tortoise iliofibularis, the tension can first be detected about 50 msec. after stimulation in winter animals (Hill, 1950c) and about 90 msec. after stimulation in summer animals (Hill, 1950a). The delay measured in this way is referred to as the *latent period.*

This method of measuring the degree of activity is unsatisfactory. The presence of the series elastic component makes it necessary for the contractile part of the muscle to shorten a certain distance before tension can be measured externally. The tension will rise in a manner determined by the stress-strain characteristics of the series elastic component and the force-velocity relations of the partly active contractile elements. Measurement of this tension will therefore give only an extremely indirect measure of the rate at which the capacity to exert tension develops in those elements. It is apparent that we need to think rather carefully about what exactly we mean by "activity" and that some way of eliminating the effects of the series elastic component must be found.

b. Satisfactory methods of measurement of mechanical effects. When a muscle is in the active state, it has the ability to shorten against a load, or to hold up a load which is just sufficiently large to prevent shortening from taking place. The degree of activity might therefore be ascertained by measuring the load that can be lifted at moderate velocities of shortening, or else by measuring the load which the muscle can sustain without

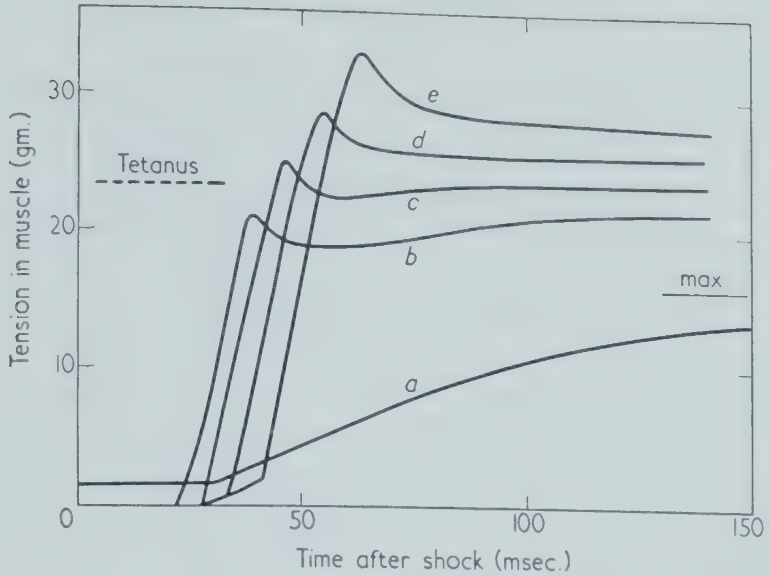

FIG. 34. The rapid development of resistance to stretch by a muscle; the curves show the effect of stretching a muscle the same amount at various times after a maximal shock. Frog's sartorius 0° C., 69 mg., 34 mm. under 2 gm.; latent period 22 msec.; 30–gm. tension equivalent to 1.5 kg./cm.^{2}. Stretch 3.1 mm. from 28.6 to 31.7 mm.; *b*, 22 to 38 msec. after shock; *c*, 28 to 44 msec.; *d*, 33–51 msec.; *e*, 42–61 msec.; *a*, for comparison, 70 msec. before shock. Broken line, final level in isometric tetanus at length 31.7 mm.; *max* denotes maximum of *a*. From Hill (1949e). (By kind permission of the Royal Society.)

either lengthening or shortening. In practice, the latter method has usually been used. This is done in one of two ways. For measurements on the initial phase of contraction, the muscle is given a quick stretch (e.g., 3 mm. in 20 msec.) which has the effect of extending the series elastic component and hence subjecting the contractile elements to a tension straight away, without them having to shorten at all (Hill, 1949c); after a suitably chosen stretch, the tension will settle down quite rapidly at the level that the contractile elements can just sustain. It should be noted, however, that the contractile elements do not necessarily have the ability to shorten under tensions just less than this. By this technique, it is found that the muscle develops a high resistance to stretch very early on in a twitch (Hill, 1949c). In Fig. 34 the effect of such stretches at various times after stimulation are shown, for frog sartorius muscle at 0° C. It will be seen that resistance to stretch reaches a value equal to the full tetanus tension at about 40 msec. after stimulation. In later experiments (Hill, 1951b), it was found that increased resistance to stretch could first be observed in frog sartorius muscle (at 0° C.) 5 msec. after stimulation.

c. Implications. The fact that the full resistance to stretch is developed in 40 msec. shows decisively (Hill, 1948, 1949c) that the signal for activity cannot be transmitted into the interior of the fiber by an ordinary diffusion process. The concentration, at the centre of a 100 μ diameter fiber, of any substance diffusing inward from the surface would, in 40 msec., have reached only a few parts in a hundred thousand of its final value. Ions such as calcium which might produce an effect at very low concentrations ($\sim 10^{-4}\,M$), are present in far too small total quantities to be able to account for the observations this way.

We have already pointed out that ability to sustain a load equal to the maximum isometric tension does not necessarily mean that the muscle can shorten against smaller loads with a velocity given by the normal force-velocity equation; it merely means that the muscle has reached its maximum inextensibility. Data on the velocity of shortening of muscle (which would have to be given quick stretches to extend the series elastic component) against moderate loads do not seem to be available for the early phases of contraction. In measurements of the velocity of shortening with very small loads ($\sim$ 20 gm.cm.^{2}) it has been found (Hill, 1951a) that the full velocity of shortening is reached by frog sartorius muscle at 0° C. in about 25 msec.; almost identical results were obtained by Abbott and Ritchie (1951). This is considerably earlier than the time at which the full resistance to stretch is encountered. As A. F. Huxley points out, the maximum shortening velocity in the particular system he describes, and also probably in other types of mechanism too, is determined by an internal resistance to shortening which depends on the degree of activation in just the same way as the muscle's ability to develop tension. Thus the velocity of shortening under zero load would, neglecting other factors, be independent of the degree of activation, and the velocity of shortening under small loads will not be very sensitive to the degree of activation. The results described above strongly suggest that this effect actually occurs in practice.

2. *Early Heat Production by Muscle*

a. Observations. The extensive experiments which have been performed to elucidate the time course of the development of activity had two principal objectives. These were (1) to decide whether or not the signal for contraction could be transmitted into the fibers by a diffusion process and (2) to investigate the relative time courses of heat production and mechanical response in the muscle, so as to learn something of the relation between the chemical and mechanical events. The first of these questions having been answered, we will now turn our attention to the second.

It is found that heat production begins at its maximum rate about halfway through the latent period (Hill, 1949d). Thus in frog sartorius muscle at 0° C., heat production can be detected 10 msec. after stimulation; in toad sartorius, 25 msec. after stimulation. In these experiments, it was not possible, for technical reasons, to stimulate the muscle simultaneously over its whole length, and the observable heat production starts off at a low rate; when corrections were made for the time taken for the propagated spread of activity, and for the flow of heat, the maximum rate of heat production was found to occur at the very start.

This initial rate is very high. In the case of frog sartorius muscle, even the observable peak corresponded to a heat output per second about ten times the activation heat (H) in a twitch; the rate then declined, reaching a plateau of about 4 H per second after 100 msec. The peak rate, of the order of 1000 gm.cm. per gram per second is even higher than the maximum rate of energy liberation (as shortening heat) during an unloaded contraction ($\sim$700 gm.cm. per gram per second), and the true peak would be higher still. If this initial heat is generated by the same reactions which occur during the shortening process, then those reactions must have a very high initial rate. This brings to mind the observation by A. Weber and Hasselbach (1954) that the initial rate of splitting of ATP by actomyosin is considerably higher than the steady rate. If the rate-limiting part of the enzymatic cycle in each case is the reactivation of the potentially active sites, as we have suggested earlier with reference to the Hill equation, then the initial burst of activity (caused by the splitting of the initial set of ATP molecules) could be explained.

b. Implications. By the time 40 msec. have elapsed after stimulation, the corrected total heat produced by a frog sartorius muscle at 0° C. is approximately 1 mcal.gm. (Hill, 1949d, from Figs. 1 and 6). If we assume a liberation of 10,000 calories of heat for each gram molecule of ATP split during this time, then the number of molecules involved will be approximately 6×10^{16} per gram of muscle. This corresponds almost exactly to the number of cross bridges in the same amount of muscle ($\sim 5 \times 10^{16}$). By the end of this time, the resistance of the muscle to stretch reaches its maximum value. That this resistance to stretch arises from the formation of cross links between the actin and myosin, when ATP is split, was suggested long ago; and the observation that this resistance becomes maximal when heat equivalent to one ATP molecule being split at each of the cross links has been liberated is in good agreement with the idea.

In frog sartorius muscle, the increased resistance to stretch is first observed 5 msecs. after stimulation, and heat production 10 msec. after stimulation (Hill, 1951b, 1949d). In toad semimembranosus, increased rigidity begins 5 msec. after stimulation, heat production after 25 msec. (Hill, 1951b, 1949d). In the iliofibularis muscle from tortoises in summer, the increase in rigidity is first detectable about 12 msec. after stimulation and heat production is first observed 60 msec. after stimulation (Hill, 1951b, 1950a). This should not lead one to suppose, however, that the mechanical effect occurred before the chemical one, for the quick-stretch technique can detect changes in extensibility which are as small as 1/2000 of the tetanus tension (see Hill, 1951b, Fig. 2) whereas the technically much more difficult thermal methods require about 1/200 of the total heat liberated before they can register (see Hill, 1950a, Fig. 2). If we examine the two phenomena on comparable scales, then the mechanical response no longer appears strikingly sooner than the thermal one. Indeed, it apparently takes a frog sartorius muscle about 15–20 msec. to develop 1% of its maximum resistance to stretch, whereas about 5–10% of the total heat in a twitch has been produced in the same period. However, a similar comparison on toad sartorius or tortoise iliofibularis shows the resistance to stretch still slightly ahead of the observed heat production, and so it is not possible definitely to decide whether these two quantities have exactly the same time courses or not. This is very disappointing, for such information could bear very directly on the fundamentals of the molecular mechanisms.

3. *Latency Relaxation*

We have seen that mechanical changes can be detected quite early in a twitch if we look for increased rigidity, but that measurements of isometric tension show no activity until after a substantially longer delay; this latent period may be 2½–4 times as long as the time for increased resistance to stretch to appear (Hill, 1951b). The series elastic element of course complicates the issue, but providing that a sensitive enough tension recorder is available, we should still be able to detect the very beginning of tension development. Such measurements, however, have not so far advanced the initial detection of tension to quite as early a moment as increased resistance to stretch appears. They do, however, reveal another phenomenon, known as latency relaxation, which may be involved in the delay.

If the muscle is initially under resting tension, the first effect of stimulation is to produce, paradoxically, a drop in tension, which begins about halfway through the latent period and is reversed by the end of it. The extent of the drop varies with the initial tension in the muscle—and so also with its initial length—in the manner shown in Fig. 35 (Abbott and Ritchie, 1951). The actual value of tension drop is very small—of the order of 1/150 of the resting tension exerted by a muscle stretched 15%, and only about 1/2000 of the maximum tetanus tension. Hill (1951b) has pointed out that these phenomena cannot be accounted for by an increase

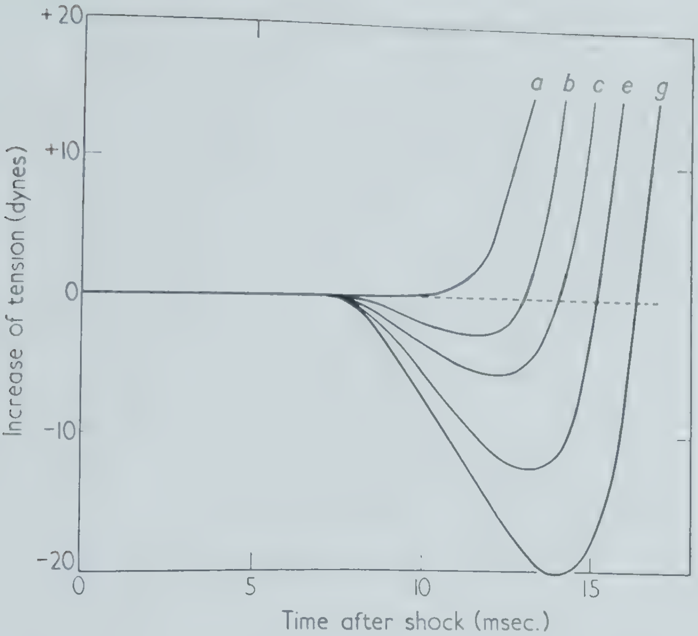

FIG. 35. The early fall of tension during a muscle twitch. Frog sartorius at 0° C., 75 mg., standard length in the body 30.5 mm. Records of twitch taken at lengths: *a*, 27 mm.; *b*, 30 mm.; *e*, 33 mm.; *g*, 35 mm. Redrawn by Hill (1951b) from Abbott and Ritchie (1951). (Reproduced by kind permission of the Royal Society.)

in extensibility during the latent period, for the stretch experiments show that some milliseconds before the earliest drop in tension, the muscle is becoming not more, but less, extensible. He has suggested a small initial lengthening (by about one or two thousandths of its length) of the parallel elastic component as a possible cause. As the elastic modulus of this component increases with length, the resultant drop in tension would be greater at longer muscle lengths, and it will take longer to reverse. This explanation fits the facts, but it was somewhat unsatisfactory in requiring an increase in length in some parallel elastic component whose properties had not hitherto been thought of as changing during a twitch.

The phenomena can be accounted for in a somewhat happier and more intriguing way by the sliding filament model. An explanation in these terms has been suggested by A. F. Huxley (1957), and has been entertained by the present author for some time too. In the model, an elastic connection across the H-zone between the ends of the secondary filaments will behave as a parallel elastic component, and any change in the structure of the system, at the appropriate time, which allows this connection to shorten slightly could produce the latency relaxation effect. Such a change could be an increase in the length of the secondary filaments, by a total amount of about 6 A. between the Z-line and the H-zone;

this small amount in each filament would give the observed drop in tension.

Again, if we were to allow repulsive forces between the filaments to contribute to the resting tension (Section IV, A), then latency relaxation could be produced by a small decrease in those forces, perhaps due to binding or release of ions.

The time course of latency relaxation shows that it begins in frog sartorius muscle at 0° C., about 7.5 msec. after stimulation, and builds up steadily until it is overtaken by the shortening of the muscle and can no longer be measured. It is therefore not possible to say how long it really goes on increasing, nor when it is terminated; but it seems to be still increasing 15 msec. after stimulation. It should be noted, incidentally, that the tension produced at each cross bridge is about twenty times as great as the drop in tension in one secondary filament associated with latency relaxation. Thus only those secondary filaments in which *no* tension has yet been generated can contribute to the latency relaxation at the beginning of a twitch.

D. The Cessation of Activity in a Muscle Twitch

1. Introduction

Although it is easier to resolve the events in time during relaxation than during activation, difficulties still remain in deciding what criteria should be adopted as a measure of the degree of activity in the muscle (Hill, 1949c, 1953d). The peak of tension in an isometric twitch occurs when the tension exerted by the series elastic component is just equal to the tension under which the contractile element will neither lengthen nor shorten. The time that the contractile element takes to stretch the series elastic component to this point in fact exceeds the period over which the contractile element can exert full tension; thus the twitch tension is still rising when the active state begins to decay and can tell us little directly about the time course of that decay.

2. Methods of Measuring the Duration of Full Activity in a Twitch

A number of ingenious methods have been developed for this purpose. If a muscle is released at a suitably chosen speed early in a twitch, the tension remains fairly steady at first while the muscle is fully active, and the moment at which tension begins to fall off may be used to define the moment at which activity begins to decline. The decay of the active state in frog sartorius muscle at 0° C. measured by this method (Hill, 1953d) begins 80–100 msec. after the stimulus, i.e., at only about one-half the time to reach maximum tension in an isometric twitch. In this method, however, the load necessarily has to be small (so that the time spent in stretching the series elastic component is short), and we have already seen that the capacity to shorten with maximum velocity under small loads is not necessarily a very good criterion of full activity; the full shortening velocity under small load at the beginning of a twitch was developed before the muscle was fully active (as judged by its resistance to stretch) and this maximum velocity may persist after activity has begun to decline. Indeed, later experiments which we will describe presently indicate that this is the case.

A second method of investigating the shape of the active-state curve has been used by Macpherson and Wilkie (1954). The curve showing the increase of isometric tension from the beginning of a twitch is compared with the same curve for the beginning of an isometric tetanus; in a tetanus, the fully active state, once reached, is smoothly maintained, and so it can be argued that when the twitch tension development curve begins to fall below the tetanus tension curve, the active state in the former is beginning to decay. The time at which this occurs in frog sartorius muscle at 0° C. is about 44±5 msec. after stimulation, somewhat shorter than that originally estimated by Hill. This method is the more sensitive one, but the result again is not entirely without ambiguity; the divergence between the two curves takes place at a time when the active state (as measured by resistance to stretch) is barely fully developed, and it may be that we are really detecting a difference in the rate of development of the active state in a twitch and a tetanus, rather than a falling away of the fully active state of a twitch. However, the error that this could introduce is only a small one.

A method for determining the entire shape of the active-state curve has been developed by Ritchie (1954b). The muscle is released a short distance at various points during a twitch, and the redevelopment of tension curve is plotted out in each case (Fig. 36). At the peak of each curve, the contractile component is neither lengthening nor shortening, and the tension it sustains can be used as a measure of the degree of activity at those points, in the same way as one can obtain a measurement of the degree of activity at a single point from the peak of the ordinary twitch tension curve. The active state curve so produced is illustrated in Fig. 37. Ritchie points out that after an average time of 340 msec. after stimulation, the intensity of the active state has fallen to 5% of its maximum, while the isometric tension exerted by the muscle was still 85% of its maximum value. As Ritchie observes, Hill (1953e) had found earlier that the production of heat during a twitch by a frog sartorius muscle at 0° C. was virtually complete 320 msec. after stimula-

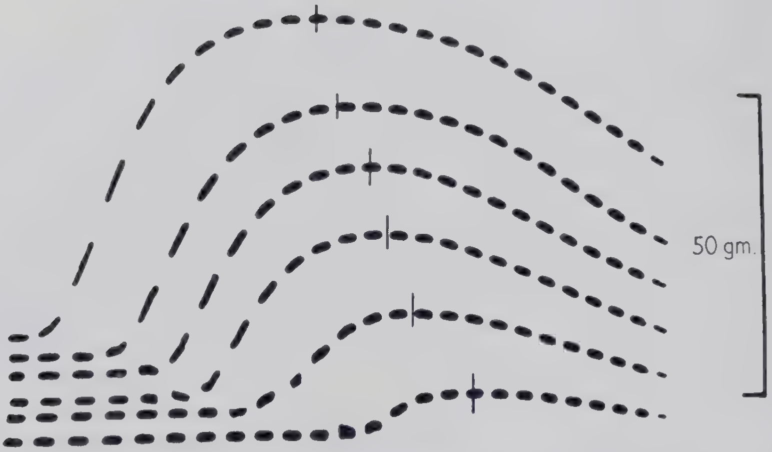

Fig. 36. Development of tension after release at various points during a twitch. Ordinate, tension; abscissa, time. Frog sartorius at 0° C. Standard length, 37 mm.; weight, 129 mg. Top record is ordinary isometric twitch at standard length. Subsequent records have been preceded by quick releases of about 3 mm. to the standard length, timed at about 10, 40, 60, 100, and 175 msec. after the stimulus. The dashes along the tension curves occur at 20–msec. intervals. From Ritchie (1954b). (By courtesy of the *Journal of Physiology*.)

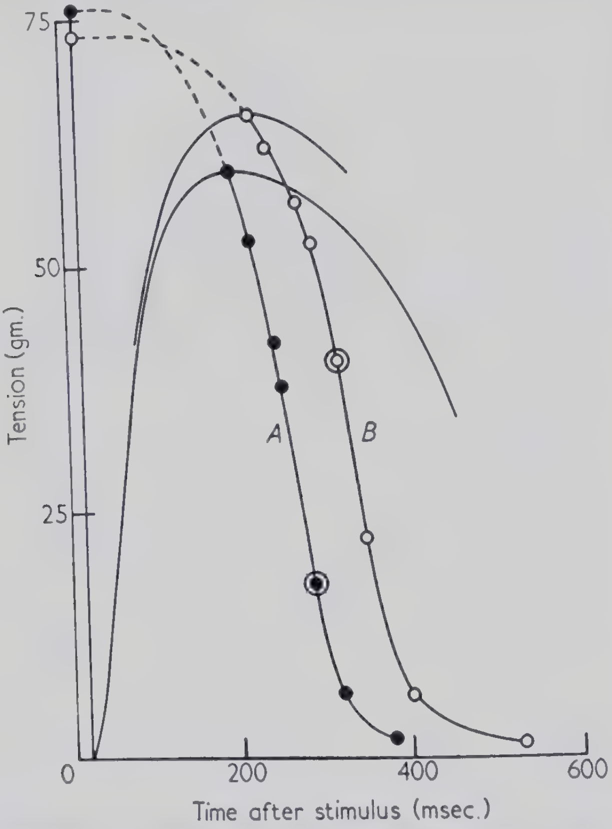

Fig. 37. Active-state curves obtained by the "release" method (Fig. 36) of frog sartorius muscle at 0° C. at standard length 34 mm.; weight of muscle, 127 mg. *Curve A:* in Ringer's solution; *curve B*: after soaking for 15 minutes in nitrate solution (see Section IV, D, 3). The interrupted part of lines mark the region of curves not obtained experimentally. The ordinary isometric twitches at the standard length are shown both for the muscle in Ringer's and in nitrate. From Ritchie (1954b). (By courtesy of the *Journal of Physiology*.)

tion: and so there is good agreement between the termination of the active state measured thermally and measured by the release method.

It was predicted by Hill (1949c) that the isometric tension during the relaxation phase would exceed the "intrinsic strength" of the contractile component (i.e., the tension under which it neither shortens nor lengthens), on the grounds that this component would be undergoing re-extension by the series elastic component. A. F. Huxley (1957) has called attention to these observations again, pointing out essentially that it may not be the process of re-extension that maintains the tension and that the effects could be produced if the contractile elements could sustain a tension at a *fixed* length after their ability to shorten had ended. Indeed, as the re-extension can only be about a millimeter at most, it is unlikely that the velocity of lengthening this would require could greatly augment a decaying tension for several hundred milliseconds. Such behavior is by no means a specific feature of the sliding filament model (a number of units, arranged in series, which could pass from a long to a short form during contraction could easily give the same effects), but it arises very naturally in that model if it is the reactivation of the cross bridges which "fails" during relaxation. The muscle stops shortening, because insufficient bridges are reactivated to maintain the tension, but the bridges which have already begun a cycle of operation are unable to complete it and therefore can continue to exert tension. The rate at which this tension decays corresponds in frog sartorius muscle at 0° C. to a half-life of the links of the order of 1 or 2 seconds. This is quite long, and one wonders whether they have been pulled out into a configuration in which, as was suggested previously (Section IV, B, 7, a), their detachment was impeded. It would be interesting to know whether the decay time varies if the muscle is released and then restretched.

In another type of experiment, Ritchie (1954b) has measured the time at which decay of tension can first be detected after the cessation of tetanic stimulation; during the tetanus, the series elastic component has taken up a steady length and so does not complicate the problem. He found this time to be 35 msec. in frog sartorius muscle at 0° C., and 12 msec. at 16° C. If activity begins to decline at the same time after the last stimulus, both in a tetanus and in a twitch, the result agrees well with that of Macpherson and Wilkie (1954), when allowance is made for the different sensitivities of the two methods. The time at which decay began was not appreciably influenced by the frequency of previous stimulation. Ritchie and Wilkie (1955) have found some prolongation of the active state after multiple stimulation, but the effect was not a very large one. This makes it unlikely that the duration of the active state is controlled by the quantity of some substance released from a substantial reservoir during the passage of the action potential. It is possible, ho ever, that stimulation releases the whole of a local concentration of so bound activating ion, say calcium, inside the muscle (cf. discussion relaxing factors, Section III, B, 2, b, ii) and that activity lasts until t ions can be recaptured again.

The maximum total energy released during a twitch is about 4 mca per gram of muscle (Hill, 1949a). This corresponds to the breakdown about five molecules of ATP on each of the cross bridges. Curiousl enough, this is about equal to the number of molecules of nucleotid bound to the actin in the muscle (Mommaerts, 1951c).

3. *Prolongation of the Active State*

It has been found (Kahn and Sandow, 1950, 1951; Sandow an Mauriello, 1953) that certain anions (nitrate, bromide, and iodide) increas the peak tension in a twitch, and it has been concluded that they d this by prolonging the active state, for tension in tetanus was not affected these results have been confirmed by Hill and Macpherson (1954) and b Ritchie (1954b). Hill and Macpherson find that the effect develops to quickly after the muscle is placed in the solution containing the ne anion for diffusion *into* the fibers to be appreciable, and the effect ca decay while the anion remains inside the fibers; it seems that it is the presence of the abnormal ions in the neighborhood of the excitable membrane which is the governing factor. In the absence of knowledge of the processes which link the passage of the action potential to the chemical events producing contraction, little can be said about how this might take place; on the basis of the previous argument, it would be the rate of recapture of the released activator which was affected.

The heat production during loaded twitches was found to be about twice as great in nitrate—or iodide—Ringer as it was in normal Ringer, and a value as high as 7.35 mcal. per gram per twitch was quoted. This seems to rule out the possibility that it is the quantity of actin-bound nucleotide which determines the maximum energy liberated in a twitch, for this quantity of nucleotide gave a correct fit for the 4 mcal. per gram of a normal twitch.

V. THE MECHANISM OF CONTRACTION

A. *Introduction*

In the preceding pages we have considered various aspects of the behavior of striated muscle in the light of the sliding filament model, and we have seen that a very generalized description of the action of the cross bridges—that they are the structural agents which cause the two sets of filaments to slide past each other, that they operate in a cyclic fashion, and that they can sustain a tension when they are attached to the secondary filaments—enables us to proceed some distance in establishing relationships between the physiological, biochemical, and structural properties of muscle. However, this still leaves us a long way from being able to say precisely what *does* happen, in molecular terms, during contraction. And it is only by doing so that one can hope to give a complete and detailed explanation of the large-scale behavior of muscles. An important step in this direction has been made by A. F. Huxley (1957) with his "vibrating sidepiece" hypothesis and the mathematical evaluation of its consequences. We shall consider some aspects of this scheme presently. First of all, though, let us consider the problem in general terms. Such considerations must inevitably contain a great deal of speculation, which can only be justified if it suggests further experiments rather than conceals the need for them.

B. General Requirements of Contraction Mechanisms

It is fairly generally agreed that the energy needed for contraction is derived in some way from the dephosphorylation of ATP and that the enzymatic properties of myosin are utilized in this process. And it appears that the quantity of energy required could probably be released by the enzyme in the time available. The structural observations give a strong indication of the maximum tension which the system could exert (from energetic considerations), and so, given the *rate* of energy release, the general range of shortening velocities of muscles can be inferred. The central problem of muscular contraction then assumes two complementary forms. It is necessary that the contractile mechanism be able to transform the energy liberated in the chemical reaction into mechanical work. And it is also necessary that the amount of mechanical work done should control the extent of the chemical reaction that takes place; for in a muscle shortening against a large load, a greater amount of total energy is liberated than in a muscle which shortens the same distance against a smaller load.

C. The Sliding Filament System

1. The Control of Energy Release

It is apparent that each enzyme site must perform a large (and variable) number of individual acts of dephosphorylation (depending on the work done) each time the muscle shortens through its normal range. The sliding filament mechanism indicates how this might happen in principle, whereas other mechanisms usually impose a fixed relationship between the length change and the amount of ATP split, and all only one molecule of ATP to be split at each enzyme site for each co plete shortening of the whole muscle. It was pointed out earlier (Secti IV, B, 1) that the energy relationships suggest that each active site only allowed to perform an act of dephosphorylation, if, at some poi during that particular cycle of its operation, it has contributed to t *tension* generated by the muscle.

In terms of a model with enzyme sites at the cross bridges betwe actin and myosin filaments, this condition requires that an enzyme si splits a molecule of ATP only if the cross bridge associated with it pe forms a cycle of operation at one phase of which actin and myosin a joined together, and tension thereby generated (though not necessari at that instant).

We should recall at this point the observed enzymatic behavior myosin (Section III, B, 2, a). At neutral pH, and in the presence of ma nesium, the ATPase activity of myosin was low, but the activity of myosi plus actin, in conditions where combination could take place betwee them, was high. Clearly there is an analogy between this behavior *in vitr* and the required enzymatic properties of myosin and actin when orga ized into filaments *in vivo*. There is, however, a slightly paradoxic element in each case. Actin and myosin are dissociated by ATP at hig salt concentrations, and even at the lower salt concentrations present i muscle, it seems that ATP must have a dissociating effect at one part o the cycle of operation in order that the cross bridges can detach fro the actin filaments and join onto them again in a new position. If AT displaces actin from myosin, how then can the actin have any influenc on the subsequent splitting of the ATP by the myosin?

We may think of the dephosphorylation of ATP by myosin as occurrin in three steps. The ATP is first bound to the myosin; the terminal pho phate band is broken; and the products, ADP and phosphate, dissociat from the enzyme. (Alternatively, one might suppose that the ADP re mained bound to the enzyme and was rephosphorylated *in situ* by phosphate-donor system.) An actin molecule might exert its acceleratin effect on any of these steps. If it is the splitting step that is influence by the actin, then one must suppose that when the system is in one con figuration, actin can perform the acceleration, while in another configura tion, ATP can displace actin without itself being immediately dephos phorylated.

This particular mechanism might fit into the sliding filament mode in the following way: The cross bridges are in one position ("out") whe a molecule of ATP is bound to them, and they move to another positio ("in"), 50–100 A. nearer the center of the A-band, when the ATP is spli and they combine with actin. When a cross bridge with an ATP molecule bound to it comes opposite the correct point on the actin filament, ATP is split, combination takes place, and the cross bridge moves from the "out" to the "in" position, drawing the actin filament along as it does so. The arrival of another ATP molecule then dissociates the actin and the myosin, and with the cross bridge in the "in" position, the splitting of that ATP molecule is not accelerated by the actin molecules in the vicinity; the cross bridge moves to the "out" position and the cycle recommences.

The mechanism proposed by A. F. Huxley embodies an acceleration of ATPase activity by actomyosin formation too, although it does so in a rather hidden way. In this system, it is the actin which is the enzyme; but the actin can acquire a molecule of ATP for splitting only if the actin has in a previous part of the cycle been combined with myosin, and if that combination has been dissociated by a molecule of ATP. Essentially, it is either the elimination of the reaction products, or the binding of ATP, which is on this scheme accelerated by actin-myosin combination.

2. *The Mechanism of Energy Transfer*

It appears, then, that the problem of regulating the rate of energy release according to the amount of work done is one where the experimental evidence does at least provide some useful clues. The problem of how the chemical energy is transformed into mechanical work is of an altogether different character. This is really the basic question in muscular contraction, and to some extent it is true to say that we have now merely succeeded in localizing the problem in the cross bridges.

If we take the view that the set of cross bridges to any particular actin filament act in parallel, then they must move a distance of 50–100 A. during each cycle of operation. This movement is associated with the splitting of one molecule of ATP; the distance involved is very large compared with the range of a chemical bond, which would exert a much larger force over a much smaller distance. To produce the required excursion, some type of lever or other magnifying system is necessary.* This is a general difficulty encountered in any contraction mechanism where a substantial number of tension generating sites on a given filament act in parallel.

An indirect means of overcoming this difficulty is to make use of Brownian motion to bring together two sites which are normally separated by too large a distance for reaction or combination to occur (H. E. Huxley, 1952). This has been done in a very ingenious way in the mechanism of A. F. Huxley. A cross bridge (or "sidepiece") vibrates to and fro in a longitudinal direction on two elastic connections, as a result of thermal agitation, until it comes near to an active point on the actin filament. Having been brought close enough in this way for short-range forces to come into operation, the sidepiece joins firmly onto the actin filament. Tension is then exerted by the stretched elastic connection, and work will be done as this shortens back to its resting length, pulling the sidepiece and the actin filament along with it. In order to make the system unidirectional, it is postulated that combination with actin can take place only with the sidepiece on one side of the equilibrium position; the combination is broken by ATP at a high rate on the other side of the equilibrium position. The immediate source of energy for contraction is the thermal motion of the sidepieces, stored as potential energy in the elastic connections, but the system does not violate the second law of thermodynamics, because energy has to be supplied to break the actin-myosin linkage in another part of the cycle.

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* E.g., the A. Szent-Györgyi (1947) "bimetallic strip" molecule.

There is very little experimental evidence at present to help one to decide how (and whether) movement of the cross bridges takes place. Pure myosin is reported (H. H. Weber, 1950; Gergeley, 1956) to be unaffected in its sedimentation constant, viscosity, and light-scattering behavior by the presence of ATP. However, changes in the shape of the molecule which could considerably alter the position of the cross bridges on the filaments might not be easy to detect with isolated myosin molecules in solution. Also, it may be that a shape change occurs only when combinations with actin takes place.

3. *Production of Constant Heat of Shortening*

The shortening heat given out by a muscle is largely independent of both length and load. It is as though shortening was always opposed by a constant force; simple friction, however, cannot provide an adequate explanation, as we have already found. The force-velocity relations in the Hill equation also give the same impression; thus one might suspect from the equation

$$(P+a)\,v = (P_0-P)\,b$$

that the rate of energy production of the system is $(P_0-P)\,b$ and that this is divided between the rate of working against the load (Pv) and the rate of working against the resistance which produces heat of shortening (av). This way of interpreting the data assumes, arbitrarily, that maintenance heat is a separate item on the balance sheet.

In A. F. Huxley's mechanism, heat production and absorption occurs at various points in the cycle at the cross bridges, but the net heat evolved is of course simply determined by the amount of ATP split and the amount of work done. With an appropriate choice of the maximum work which one cross bridge can perform per cycle (which of course has to be less than the energy released from one molecule of ATP), it was found that the predicted heat production agrees well with experimental observations; that is, choice of variables which gives a constant heat of shortening also gives close agreement between the thermal and dynamical values of *a*. The system has a "built-in" tendency to produce a constant heat of shortening; for when the load is increased, more enzyme sites are functioning and the energy released for a given amount of shortening increases; but because the system is shortening more slowly, it operates more efficiently and a smaller proportion of the energy available from each of the active sites is wasted as heat.

In this mechanism, the agreement between the thermal and mechanical values of *a* is accidental. However, a number of the constants involved are probably not entirely independent of each other (e.g., reaction rate constants and the work and energy terms); and so a concealed requirement that the two values of *a* should be similar may exist.

On the other hand, it seems to the present author that one would expect a constant ratio to exist between the maximum tension (P_0) and the heat of shortening (*a*) at all muscle lengths, if the latter is simply a fraction of the total energy being released which has to be wasted. Perhaps the efficiency of the process decreases at shorter muscle lengths, but it is a remarkable coincidence then that the over-all result should be a heat of shortening of such constancy as that found by Abbott (1951).

4. *The Role of Actin in Contraction*

In contrast to its apparent lack of effect on myosin, ATP seems to be involved in at least two important structural reactions in actin, and it would be surprising if these did not play some part in the sliding process. The monomer-dimer transformation of actin, which Tsao (1953b) showed took place instantaneously when ATP was added to actomyosin is a very striking phenomenon indeed, and the dephosphorylation of bound ATP which accompanies the G → F transformation of actin (Section III, B, 1, b) could also provide a link between chemical and mechanical effects. One could speculate that if these transformations, or equivalent ones *in vivo*, produced small changes in the length of the actin filaments (the longitudinal integrity of the filaments being maintained by the "backbone" material), it might be possible to make the actin filament crawl along the system of cross bridges from the three myosin filaments around it.

VI. Conclusion

The purpose of this chapter was to see to what extent our present knowledge of the molecular structure of muscle can illuminate the relation between the physiological and the biochemical properties of the tissue. The sliding filament model, and its detailed dimensions, now seem fairly well established. This model gives a good qualitative picture of many phenomena in muscle. It also allows us to make satisfactory quantitative estimates of a number of parameters involved in contraction—for instance, the maximum tension, the order of magnitude of the shortening velocities, the time for full activity to develop—on the basis of structural and biochemical measurements. And there are indications of how the system is constrained to release its energy in an efficient manner. But the most interesting problems remain unsolved. The mechanism of energy transfer from the chemical to the mechanical form is unknown. So also are the limiting factors which govern the power and speed of contraction. These factors are related to the maximum velocity at which the relevant enzymatically catalyzed reaction can take place, and to the minimum size that the enzyme molecule or system needs to have. As such, they are part of a more general problem, in which advances in one field should assist understanding in many others.

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